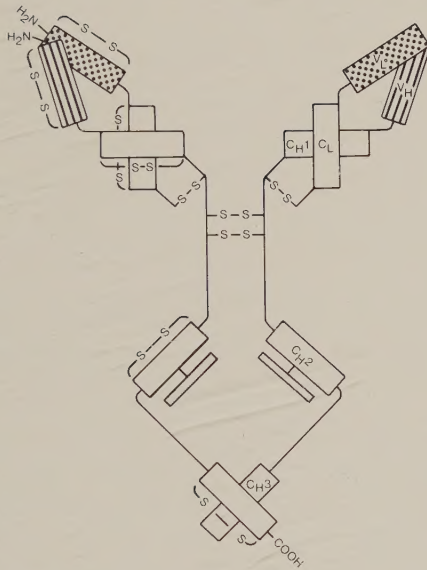


CONFORMATIONAL DEVIATION IN IMMUNOGLOBULINS RELATED TO IMMUNE COMPLEX FORMATION IN RHEUMATOID SERA

Michael Uesson



Lund 1983

CONFORMATIONAL DEVIATION IN IMMUNOGLOBULINS RELATED TO
IMMUNE COMPLEX FORMATION IN RHEUMATOID SERA

by

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Abstract Hidden IgM RF, firmly associated with autologous IgG and exhibiting no detectable RF activity, cannot be detected by conventional RF tests. Quantitative precipitin analysis using rabbit IgG anti-human Fc_u is proposed as an alternative method of detecting hidden, as well as free IgM RF directly in serum. All IgM RF analysed produced deviating precipitin curves in the antigen excess zone, compared to those of normal IgM. This could be explained by an increased hydrophobicity due to the interaction of RF in secondary immune reactions, such as re-arrangement of immune complexes and multiple bonding within the complexes. The formation of immune complexes in sera containing free or hidden IgG RF could only partially be explained by a self-association of IgG RF. Two affinity chromatography systems, utilizing the immobilized F(ab')₂γ of two different specific rabbit IgG, were employed to isolate minor populations of IgG, intermediate complexes, and rapidly sedimenting complexes all of which contained unique determinants unrelated to RF. By circular dichroism (CD) it was shown that these unique determinants, which in one case were located to the F(ab')₂γ, were related to certain conformational deviations in the monomeric immunoglobulins, possibly reflecting a special antibody specificity. The pool of IgG-IgG complexes was isolated from rheumatoid serum and investigated by CD analysis. The monomeric IgG derived from these complexes had a deviating conformation which was related to the complex-forming properties. Monomeric IgG of the same origin, which did not form complexes, had a normal conformation. IgG-IgG and IgG-IgM complexes with unique determinants were isolated from rheumatoid serum by means of a third affinity chromatography system utilizing the immobilized F(ab')₂γ of a third specific rabbit IgG. The monomeric IgG derived from these complexes formed new IgG-IgG complexes, which all contained the unique determinants. The conformational deviations in CD analysis, earlier observed for the pool of IgG-IgG complexes, could now be related to the presence of unique determinants, which in turn were related to an increased optical activity of certain hydrophobic amino acids, and to the complex-forming properties. These unique determinants were present in one third of the Faby derived from the IgG molecules, and this population of Faby had a deviating conformation as analysed by CD, compared to the other population of Faby without the determinants. A model is proposed to explain the physiological occurrence of these new markers in rheumatoid serum. The unique markers are involved in the formation of immune complexes and can be useful instruments for further investigation.			
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Lund 1983

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PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by the appropriate Roman numerals.

- I. Uesson M and Hansson U-B. Quantitative precipitin analysis of free and hidden RF IgM. Scand J Immunol 14, 61, 1981.
- II. Hansson U-B, Uesson M and Alkner U. Isolation and characterization of intermediate complexes and other components with common antigenic determinants. Scand J Immunol 13, 57, 1981.
- III. Alkner U, Uesson M, Hansson U-B, and Glans J. Circular dichroism of immune complexes with rheumatoid factor activity. Mol Immunol 19, 21, 1982.
- IV. Uesson M and Hansson U-B. Circular dichroism of immune complexes, IgG and Fab γ with unique determinants from rheumatoid serum. Scand J Immunol 16, 249, 1982.

Rheumatoid factor (RF) and immune complexes (IC) in the circulatory system are central aspects of molecular research in the pathogenesis of the rheumatoid diseases. Specific methods are needed to isolate the immune complexes and to separate their constituent molecules in antibody and antigen. Knowledge of the conformation of the constituent molecules and their biological activity, and of the intact immune complexes may shed light on the mechanisms underlying complex formation and on the immunopathological role of immune complexes. Further information about the activity and structure of RF and IC is also needed for the elaboration of more specific and effective methods of diagnosing rheumatoid diseases. Improved information of this kind could possibly be used in selecting specific molecules for in vitro or in vivo interaction with immune complexes and related immunoglobulins in the circulatory system of patients with rheumatoid diseases.

The aim of the studies outlined in this thesis has been to elaborate a new method for the detection of rheumatoid factor in whole serum, whether free or hidden in immune complexes, and to isolate and characterize immune complexes in rheumatoid serum and their constituent molecules with regard to their biological activity and conformation.

The following investigations were made (Roman numerals refer to respective publications - see p 9):

I. The quantitative precipitin reaction of free or hidden (in immune complex) IgM RF with rabbit IgG anti-Fc μ was investigated in order to explain deviations in precipitation compared to that of normal IgM. Some of the mechanisms behind the reactions were studied. In order to establish whether the new precipitin test could be used to detect both free and hidden IgM RF directly in rheumatoid serum, quantitative precipitin analysis was run in parallel with the convent-

ional Waaler-Rose test for routine use and a modified Waaler-Rose test for the detection of hidden RF.

II. Immune complexes and related immunoglobulins with RF-activity were isolated from rheumatoid and related sera. The isolated components were then characterized with regard to RF activity, dissociation and association of the complexes, IgG subclass distribution, sialic acid content, presence of unique antigenic determinants (unique conformation) and sedimentation properties.

III. IgG-complexes were isolated from rheumatoid serum and characterized by means of circular dichroism. The conformation of the intact IgG-complexes and of their constituent molecules was compared with that of non-complexforming IgG from rheumatoid serum and normal serum.

IV. IgG-IgG and IgG-IgM complexes containing unique antigenic determinants were isolated from rheumatoid serum and characterized by means of circular dichroism. IgG was isolated from the complexes and Fab γ fragments were derived from the IgG. The conformation of the complexes, of isolated IgG and Fab γ fragments was investigated.

INTRODUCTION

Immunoglobulins

The highly organized mammalian immune system comprises a multitude of specialized cells and molecules which provide protection against invasion by pathogenic organisms. The humoral immune system includes the B cells which can be induced to proliferate and secrete specific immunoglobulin molecules, called antibodies, which in turn may react with, for example, invading organisms or molecules called antigens, in the circulatory system. The potential capacity of the humoral immune system for recognizing specific molecular structures in antigens is enormous. It has been calculated that 10^8 specific antibodies, each with a different specificity, can be synthesized in the mouse (Todd et al, 1982).

The immunoglobulins are glycoproteins composed of equal numbers of light ($M = 22,000 - 23,000$) and heavy ($M = 51,000 - 76,000$) polypeptide chains which are united by interchain disulphide bonds and non-covalent forces. In immunoglobulins, class is determined by the nature of the heavy chain, five classes of heavy chains being known: IgG(γ), IgM(μ), IgA(α), IgE(ϵ) and IgD(δ). They differ in the amino acid sequence of the constant C_H part of the chains, the molecular weight, the carbohydrate content, and in certain biological effector functions. Immunoglobulin classes can be subdivided in subclasses, which differ in the amino acid sequence and the molecular weight of the heavy chain. There are, for example, four subclasses of IgG, differing with regard to the number and location of the disulphide bonds between their heavy chains. In addition, these subclasses may have different biological activity. The light chains exist in two forms, κ and λ ; like the heavy chains they can be subdivided into special groups on the basis of variations in the amino acid sequence of the constant portion of the chains. Any immunoglobulin molecule contains one type of heavy chain and one type of light chain; IgG, for example, containing two γ chains and two κ or λ chains, can be re-

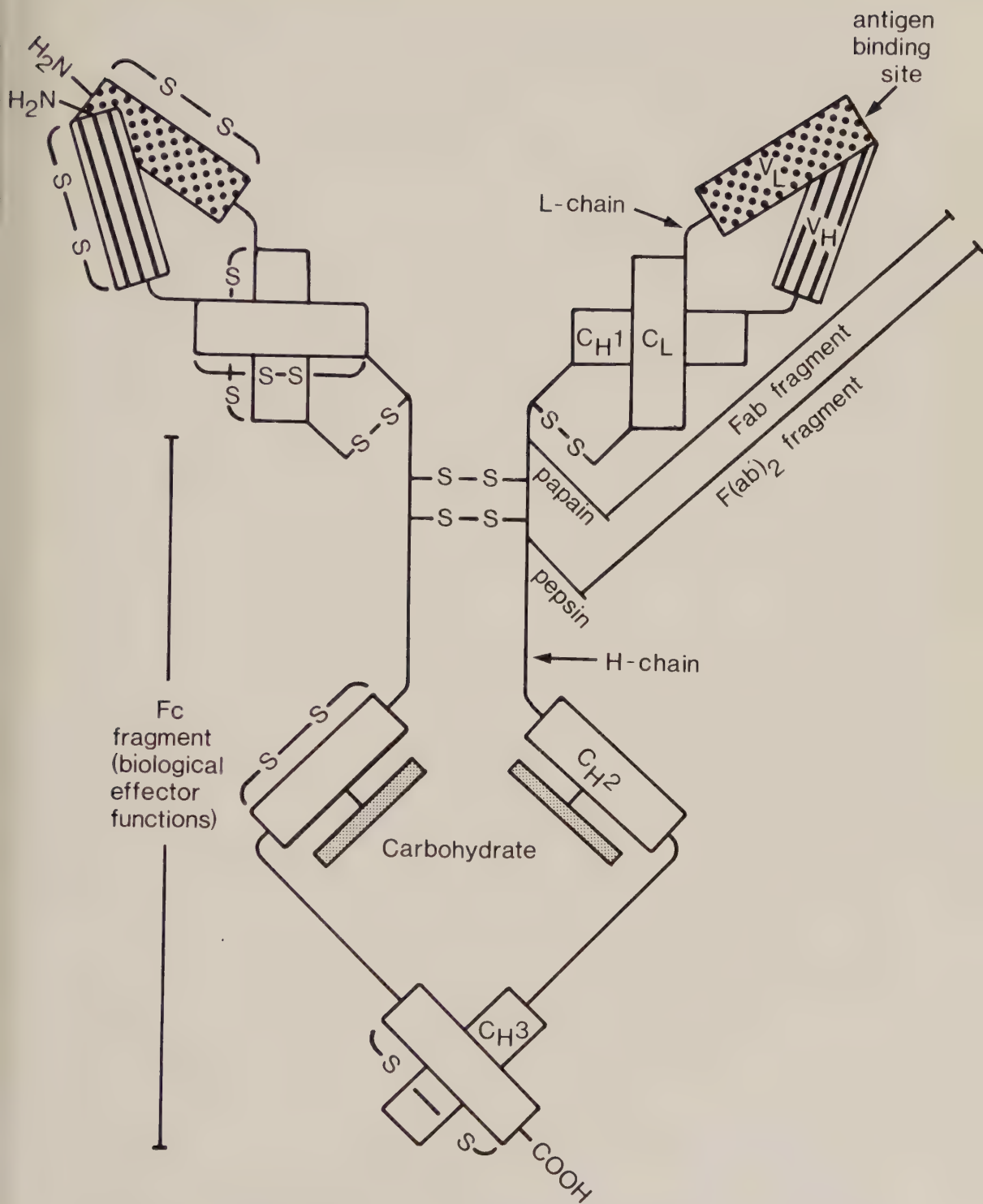
garded as a symmetric molecule composed of two identical half-molecules united by disulphide bonds between the γ chains.

Each chain is folded into domains of similar dimensions (110 - 120 amino acids). In IgG, all domains, except C_H2 , are arranged in pairs by non-covalent forces, each domain being held together by intra-chain disulphide bonds. Whereas light chains have two domains, heavy chains have four to five domains, depending on the class of the chain. The N-terminal variable domain (V-domain) of any chain varies substantially in amino acid sequence, as compared with the other domains (C-domains) of the chain with more constant amino acid sequences. The amino acid sequence of V-domains may, however, be partially restricted, thus different immunoglobulin molecules may belong to the same V-region subgroup. The antigen binding site is characteristic for each individual antibody molecule, and is located in the hypervariable region of the V-domains of the light (V_L) and of the heavy chain (V_H). Molecular structures which are located in the V-domains and related to the antibody activity of an individual antibody molecule are called idiotypic.

IgG, IgM and IgA contain genetic markers, allotypes, characterized by inherited amino acid substitutions in the constant C-regions of both the H-chains and the κ light chains.

The carbohydrate content of IgG

IgG, like the other immunoglobulin classes, is a glycoprotein and contains 2 - 3 % carbohydrate irrespective of subclass (Nisonoff et al, 1975a). The major portion of the carbohydrate is mainly localized to the Fc part of the γ chain, but the Fd part of the γ chain and the light chains can also contain carbohydrate. Characteristically the carbohydrate is in the form of a branched oligosaccharide chain which contains mannose, galactose, fucose, N-acetylglucosamine and sialic acid. A recurrent structure in oligosaccharides is a single chain of two residues of N-acetylglucosamine, one of which is bound to an



Schematic diagram of an IgG molecule of subclass 1, adapted from Marquart and Deisenhofer (1982).

asparagine side chain of the amino acid backbone of the γ chain, while the other is bound to mannose, which is a branch-point to which two other chains, each of two to four monosaccharide residues, are bound (Nisonoff et al, 1975b). Galactose is at the penultimate or terminal position in the branches, whereas sialic acid, when present, always ends the chains. There is a considerable heterogeneity in the carbohydrate content of IgG, and the amount of hexose in IgG has been shown to vary from 9 - 37 moles of hexose per mole of different myeloma IgG (Abel et al, 1968). In addition, IgG exhibits micro-heterogeneity, largely confined to the outer branches of the oligosaccharide chain, and IgG molecules with two, one or zero sialic acid residues exist in individual myeloma IgG (Kornfeld et al, 1971). Normal IgG contains two oligosaccharide chains linked to asparagine (Mizuochi et al, 1982), i e one oligosaccharide per heavy chain.

Rheumatoid disease

Rheumatoid arthritis is a systemic disorder affecting not only the joints but also several organ systems and rheumatoid disease has been proposed as a collective term. Rheumatoid diseases are wide-spread, affecting two to three percent of the population and, in Sweden, accounting for as much as one sixth of the total expenditure for diseases (Herzman, 1979). Rheumatoid diseases are difficult to diagnose at their onset, and the prospects for most chronic cases are rather poor, despite the availability of medicines capable of alleviating both pain and symptoms.

Rheumatoid disease is assumed to be an auto-immune disorder and can be divided into several clinical syndromes by a number of criteria (Mitchell and Fries, 1982). One such criterion is the presence of rheumatoid factor (RF) in serum.

Rheumatoid factor activity

Circulating immunoglobulins of IgM, IgG and IgA classes capable of

reacting with autologous, homologous or heterologous IgG, are characteristic of rheumatoid arthritis (RA) and are collectively termed rheumatoid factors (RF). RF is thought to be an auto-antibody that can react with the Fc part of the various IgG.

RF can be detected by the use of tests based on the antibody reactivity of RF to human or rabbit IgG. Hidden RF is bound to autologous IgG, though the bond is a weak one, since the hidden RF can be detected by conventional serological tests only after dissociation from IgG by gel filtration at neutral pH (Bluestone et al, 1969), or after the addition of EDTA, NaCl and glycine at neutral pH (Hansson and Winblad, 1978). When the hidden RF is strongly bound to autologous IgG, treatment of serum with solvents at acid pH (Falus et al, 1979), or gel filtration at acid pH (Moore et al, 1978), may facilitate their detection. That strongly hidden RF may remain undetected is indicated by the variation in frequency of hidden RF in sero-negative RA (Hansson and Winblad, 1978).

The heterogeneity of the RF antibody activity is demonstrated by reactivity with several IgG determinants in IgG of different origin. The binding constant between RF and human 7S IgG is low, in the order of 10^2 to 10^3 l/mole (Dissanayake et al, 1979) or 10^4 l/mole (Eisenberg, 1976). Various determinants located in the C-domains of the IgG molecule have been proposed as possible antigenic reaction sites for RF (reviewed by Johnson and Faulk, 1976). Though RF may sometimes react with normal human IgG whose structure is assumed to be native in vitro, certain RF react preferentially with mildly reduced and alkylated IgG; this has given rise to the proposition that the reaction between RF and normal IgG is, in fact, a cross-reaction, and that the true reaction is one between RF and an IgG that is conformationally deviant in vivo (Brown and Brown, 1981, 1982). This has received support from the observation that, in the reaction of IgM RF with rabbit IgG, the antigenic determinants are similar to those of heat-denatured human IgG which do not occur in native

human IgG (Gaarder and Michaelsen, 1974), but may occur in certain immune complexes (Arai and Shimizu, 1981).

RF reacts with immune complexes (Tesar and Schmid, 1970), even with those produced in the normal immune defense against invading non-physiological molecules (Markenson et al, 1975). RF influences the size of, and is capable of aggregating, immune complexes (Edelman et al, 1958), but can even solubilize certain of them (Alkner and Hansson, 1979). The size of immune complexes may influence their biological properties; thus, for example, small-latticed immune complexes in general, containing one or two antibody molecules, are removed more slowly from the circulation than larger complexes (Mannik, 1980). Other immune complexes with which RF may react are those formed between idiotypic and anti-idiotypic antibodies (Morgan et al, 1979). Such complexes may, for example, occur in humans as a result of the synthesis of specific idiotypic anti-tetanus antibodies following immunization with tetanus toxoid (Geha, 1982). It has been proposed that RF may be directed primarily against new antigenic determinants created or exposed by the formation of immune complexes (Nemazee and Sato, 1982).

Heterogeneity is also manifested by cross-reactions with, for example, cell nuclear components. It has been shown that the total amount of polyclonal IgG RF in a rheumatoid serum cross-reacted with cell nuclear antigens (Hannestad and Johannessen, 1976). This cross-reactivity was inhibited by human IgG Fc fragments, supporting the notion that there could exist an antigen to RF in vivo which was structurally related, but functionally unrelated, to IgG (Hannestad, 1978). Cross-reactivity with cell nuclear antigen has also been reported in the majority of IgM RF studied (Johnson, 1979); more specifically in the case of one polyclonal IgM RF it has been shown that the cross-reaction was with DNA-histone (Hannestad and Stollar, 1978). The relative binding affinity of a monoclonal IgM RF to DNA-histone has been shown to be lower than that to monomeric human IgG, indicating that neither of the antigens was the specific one and

that the observed reactivity might even have been coincidental (Agnello et al, 1980). The specific antigen to RF in vivo may be present in serum at very low concentration compared to the abundance of irrelevant IgG with which RF is capable of cross-reacting. It has been suggested that RF with high affinity to IgG reacts locally at the site of synthesis with autologous IgG of unknown structure, precipitating in the tissues and never reaching the circulatory system (Robbins et al, 1978). It is thus possible that RF affinity for specific antigens is much higher in vivo than when measured with various IgG in vitro.

Rheumatoid factor structure

Both monoclonal IgM RF (Kunkel et al, 1974), polyclonal IgM RF, and IgG RF are enriched in κ light chains (Carson and Lawrance, 1978a), while polyclonal IgG RF predominantly comprises IgG of subclasses 1 and 4 (Shakib and Stanworth, 1978). Monoclonal IgM RF shows restrictions to the V_{κ} III subgroup of the variable portion of the κ light chain (Kunkel et al, 1974), while polyclonal IgM RF has been shown to be restricted to the V_H III subgroup of the variable part of the heavy chain (Førre et al, 1977).

The first report of restrictions related to the antibody combining site of RF came in 1973, when cross-idiotypic determinants were demonstrated in isolated monoclonal IgM RF (Kunkel et al, 1973). Common idiotypic determinants of two monoclonal IgM RF, isolated from genetically dissimilar individuals, were located in hypervariable regions with virtually identical amino acid sequences (Capra and Klapper, 1976). The hypervariable regions, the idiotypic determinants, and the antibody combining site seemed to involve the same molecular structure, indicating that the complementary conformation of the postulated IgG antigen would be the same for both IgM RF.

Subsequently cross-idiotypic determinants occurring on monoclonal IgM RF were also described in RA sera containing polyclonal IgM RF (Carson and Lawrance, 1978b). Cross-idiotypic determinants of monoclonal IgM RF also occur in monoclonal IgG RF (Førre et al, 1979), showing that such determinants are not specific to IgM. Several polyclonal IgM RF may exhibit the same cross-idiotypic determinants that occur on monoclonal IgM RF (Førre et al, 1979) which suggests that the determinants involved are limited to certain conformations and related to the RF activity. If these specific conformations of unrelated RF molecules reflected a common antibody combining site, then the complementary conformation of the specific antigen to RF would also be limited to a specific molecular arrangement. It is of interest to note that, since the cross-idiotypic determinants of IgM RF may be inherited, they may be present in serum before the appearance of any clinical symptoms of rheumatoid arthritis (Pasquali et al, 1980).

Rheumatoid factor synthesis

In mice *in vivo* (Izui et al, 1979) and in human peripheral blood lymphocytes *in vitro* (Slaughter et al, 1978), RF synthesis can be induced by polyclonal B cell activating substances. It is assumed that, by interacting with non-immunoglobulin receptors on B cells, the activating substances cause the cells to divide and differentiate into antibody-secreting cells without specific immunization, and that the physiological synthesis of RF may be the result of such induction (Möller et al, 1980).

However, there are also indications of a more specific process in the synthesis of RF, the same cross-idiotypic determinants having been detected in polyclonal IgM RF and in the autologous B cells (Dobloug et al, 1979). These determinants occurred in both polyclonal and monoclonal IgM RF in homologous sera (Førre et al, 1979). This indicated that also the B cells in homologous sera were capable of expressing a limited number of cross-idiotypic determinants related to

RF, and thus be constituted of a more specific population of cells than would be expected from polyclonal B cell activation.

Epstein-Barr virus (EBV), the T cell independent polyclonal B cell activator, has been found to induce greater synthesis of RF in B cells isolated from RA patients than in those of normal individuals (Carson et al, 1979). (This, considered in conjunction with the presence in RA patients of antibodies against certain EBV transformation antigens, has given rise to speculation that EBV may be involved in the aetiology of RA (Vaughan, 1979).) Pokeweed mitogen (PWM), a T cell dependent polyclonal activator, induces the synthesis of larger quantities of RF from a substantially higher proportion of B cells from RA serum than from normal serum, indicating that T cells are necessary to RF synthesis in RA (Koopman and Schrohenloher, 1980). It has since been verified that those B cells in RA that respond to induction by EBV *in vitro*, mainly synthesize IgM RF bearing idiotypes which are not expressed on IgM RF molecules *in vivo*. By contrast, IgM RF molecules *in vivo* are exclusively synthesized by the limited population of B cells responsive to the T cell dependent induction by PWM (Pasquali et al, 1981). The synthesis of specific RF molecules present *in vivo*, requires the active participation of T cells, which would also be the case if specific RF were synthesized by the mechanisms involved in active immunization. The relevance of such mechanisms *in vivo* is indicated by recent *in vitro* experiments (Pisko et al, 1982) showing that normal human peripheral blood mononuclear leukocytes can be induced to synthesize RF in response to polyclonal stimulation by heat-aggregated human IgG.

Immune complexes

In general, the term "immune complexes" is a broad concept embracing all complexes formed by interaction between antibody and antigen. Immune complexes occur in the serum of healthy individuals (Paganelli et al, 1979; Delespesse, 1980; Puskás et al, 1982), and possibly function in the immune regulation (Theofilopoulos, 1980;

Taylor, 1982). The activity and structure of immune complexes must be carefully characterized, in order to assess their function in health and in disease, and to design appropriate diagnostic methods.

Methods currently in use for immune complex detection utilize a variety of principles. This causes large discrepancies in the quantitative and qualitative sensitivity of the methods, as was shown in a WHO investigation when eighteen methods for immune complex detection were compared (Lambert et al, 1978). Furthermore, to permit the comparison of immune complex analyses, and that of results from different laboratories, the standard of aggregated IgG must contain a constant concentration of IgG aggregates of comparable size (McCarthy et al, 1981).

Immune complexes in rheumatoid disease

Circulating immune complexes (IC) occur in serum from individuals with rheumatoid arthritis (Kunkel et al, 1961; Pope et al, 1975; Roberts-Thomson et al, 1976; Halla et al, 1979; Bourke et al, 1982). According to present concepts the IC are involved in the pathogenesis of RA (Ziff, 1973; Zvaifler, 1974; Harris, 1982) and trigger several molecular and cellular mechanisms causing inflammation and joint destruction (Carter, 1973; Harris, 1982). The presence of IC in serum is correlated with the presence of RF (Hay et al, 1979; Levo et al, 1981; Pope et al, 1981; Mallya et al, 1982). IgM RF (Yoshinoya et al, 1982) and IgG RF (Pope et al, 1975; Gabriel and Hadler, 1981) or both (Jones et al, 1980) are present in the complexes. The concentration of circulating IC seems to be a useful index of disease activity (Levo et al, 1981; McDougal et al, 1982), and results from long term studies suggest that IC are one of the first immunological abnormalities to appear in the circulation of RA patients (Jones et al, 1981), and may precede the clinical diagnosis of the disease.

RF and IC are also present in the synovial fluid and in paired samples it was shown that a higher frequency of RA synovial fluids contained

an increased quantity of IC compared to the corresponding serum samples (Halla et al, 1979; Hay et al, 1979). Although it has been shown that IgM RF, as well as IgG RF, are synthesized locally by rheumatoid synovial membrane plasma cells (Cecere et al, 1982), this does not necessarily exclude RF synthesis elsewhere in the body. There are indications that synovial synthesis of RF begins after systemic synthesis has been amplified, and that immune complexes occur in the synovium prior to RF synthesis (Jones et al, 1982).

MATERIALS AND METHODS

General

All information pertaining to Materials and Methods used in the experimental work can be found under the corresponding sections in the four publications. Bearing in mind that the RA sera OS and ED are referred to as R1 (OS) and R2 (ED) in paper I, and as OS and ED in papers II, III and IV, the results of paper I (showing that IgM RF of both OS and ED produced deviant precipitin curves) may be compared with those of papers II, III and IV (which characterize immunoglobulins and immune complexes related to RF).

Circular dichroism

Circular dichroism (CD) is a physicochemical method for measuring the wavelength dependence of the differential absorption of right and left circularly polarized light of a chromophore. CD is useful in studies of the structure of proteins, whose characteristic CD spectra are obtained by the spatial asymmetry of amino acids in the macromolecular backbone. The CD spectrum of a large protein molecule will be dependent on a number of optically active amino acid residues with characteristic molar extinction coefficients (ϵ_L and ϵ_R) for left and right circularly polarized light. For each wavelength, in the regions of absorption, there will be differential absorption of L and R circularly polarized light, the difference $\epsilon_L - \epsilon_R = \Delta\epsilon$ being termed the CD. The CD is measured experimentally in a spectro-polarimeter and is usually translated to the ellipticity ($\theta = 3300 \Delta\epsilon$). The mean residue ellipticity, used mainly in protein analysis, is calculated as: $[\theta]_\lambda = \theta \cdot M_0 / c \cdot l$, where c is the protein concentration (g/100 ml); l the pathlength in decimeters; M_0 the mean residue weight taken as 110; and θ the recorded ellipticity in degrees. A plot of θ (y-axis) against the wavelength (x-axis) is called a CD spectrum. At each wavelength, CD bands can be positive or negative, the magnitude of each band representing the degree of asymmetry.

Although CD spectra of proteins in general are difficult to interpret in terms of detailed molecular structure, CD is very useful in comparisons of the overall protein conformations of similar proteins which are expressed as the average CD of a large number of structures which altogether contribute to the total protein conformation. Detailed interpretation of CD spectra is complicated by symmetric chromophores being asymmetrically surrounded by neighbouring groups and by the fact that the peptide bond, which is the principal structure of the protein backbone, may assume any one of a variety of conformations depending on the surrounding structures. In terms of molecular structure, CD can be interpreted with homogenous proteins, whose exact structure is known. A major use of CD has been in the analysis of conformational changes of proteins, for example, enzyme structure in relation to substrate or coenzyme binding, protein denaturation and protein folding.

Throughout this investigation polyclonal (non-homogenous) immunoglobulins have been analysed by CD. CD was used to compare the conformation of immunoglobulins with different antibody activity or different exposure of unique structures detectable by specific rabbit antibodies directed against the molecules. The CD spectra obtained were directly comparable and any deviations in the CD spectra would be related to conformational differences between the immunoglobulin molecules. Such conformational differences were observed between different immunoglobulin molecules in identical solvents, or between identical immunoglobulin samples in different solvents. The regions of special interest in comparing the structure of, for example, two different polyclonal IgG, have been those in the near ultraviolet region between 250 - 300 nm. The intensities of transitions in this region are related to the exposures of aromatic amino acids, especially tryptophan, and disulphide bonds in asymmetric environments.

RESULTS AND DISCUSSION

Quantitative precipitin analysis of free and hidden RF IgM (I)

The purpose of this investigation is explained in the introduction to the publication I. The primary issue was to elaborate a method for the detection of hidden RF, without RF-activity, when bound in immune complexes in RA serum. There already existed a variety of methods for the determination of hidden RF after it had been released from the complexes. The new element in this investigation was to try to detect RF when present in complexes, regardless of the strength of the bond. Furthermore, the investigation was so planned that information would be obtained about the mechanisms behind the binding of RF to immune complexes. For diagnostic purposes a technique is needed to detect strongly hidden RF, whose activity cannot be detected in conventional tests, and that cannot be dissociated without risk of denaturation. Also, as previously mentioned (see immune complexes in rheumatoid disease), since the presence of immune complexes may precede that of detectable free RF, it may be important to the early diagnosis of rheumatoid disease.

It was originally indicated in an early report (Kunkel et al, 1959) that, compared to normal IgM, IgM RF produced a deviant precipitin curve with anti-IgM, giving more precipitates in the antigen excess zone and appearing less soluble. A survey of the limited work reported in the literature suggests that decreased solubility in the antigen excess zone could be related to the number of antigenic determinants, to the molecular weight of the antigen, or to the purity of the antigen. In a systematic study of a number of IgM, we found that the quantitative precipitin analysis utilizing specific rabbit IgG anti-human Fc μ , could be used to distinguish IgM RF from normal IgM. In the antigen (IgM) excess zone, more precipitates formed with IgM RF, even when hidden, than with normal IgM; this difference was so pronounced that analysis of IgM in three concentrations (corresponding to the zones of antibody in excess, slight antigen in

excess, and higher antigen in excess) could be used to establish the presence of IgM RF in whole serum. In the zones of antibody excess and slight antigen excess IgM RF did not react with IgM/anti-IgM complexes owing to its anti-IgG activity but reacted with the IgG anti-Fc μ in the same way as normal IgM. In the zone of a higher excess of antigen, more precipitates formed for IgM RF than for normal IgM, and this was the only zone of interest in discussing the differences. This was also in agreement with the interaction of RF and immune complexes being most obvious in the antigen excess zone (Edelman et al, 1958; Tesar and Schmid, 1970).

There were several possible explanations to the increased precipitation of both free and hidden IgM RF in the zone of high antigen in excess.

1. That IgM RF reacted with IgM/anti-IgM complexes

Both free and hidden IgM RF bound to the IgM/anti-IgM in this zone. Hidden RF reacted as a result of a rearrangement of the complexes containing hidden RF, which in turn caused exposure of RF activity and preferential binding to immune complexes containing rabbit IgG anti-Fc μ .

The interaction of RF with the immune complexes consisting of IgM and rabbit IgG anti-Fc μ , had to be associated with a rearrangement of the complexes, as the corresponding precipitates obtained with IgM RF and normal IgM contained equal amounts of IgM. IgM RF can rearrange immune complexes of a certain composition in the antigen excess zone, causing the release of antigen (Alkner and Hansson, 1979). Thus IgM RF was incorporated in the complexes in exchange for other IgM molecules. This rearrangement of the complexes resulted in a lattice structure having a decreased solubility in water.

The decreased solubility, when IgM RF was incorporated in the pool of IgM/anti-IgM complexes, depended on multiple bonds between the

molecules in such complexes. In the complexes containing IgM RF, bonds may exist between the Fab μ part of IgM RF and either the Fc γ part of autologous IgG, or the rabbit IgG anti-Fc μ , or both; in addition, that is, to the known bonds between the Fab γ part of the rabbit IgG anti-Fc μ and the Fc μ part of the IgM molecule. Only the latter type of bonds could occur in the precipitates obtained with normal IgM.

2. That IgM RF had a deviant conformation

Conformational deviation could have been related to antigenic determinants in the Fc μ part of the IgM molecule available for reaction with the rabbit IgG anti-Fc μ . The conformation of the Fc μ part of IgM is affected when Fab μ is bound to antigen (Brown and Koshland, 1977; Chiang and Koshland, 1979) and possibly this might also be the case with IgM RF. Thus, a conformational deviation could have occurred in the Fc μ part of IgM RF, when IgM RF formed Fab μ -Fc γ bonds with rabbit IgG anti-Fc μ , or when hidden IgM RF reacted with the Fc γ of autologous IgG. Such a conformational deviation might either be directly related to the solubility properties of the RF when present in immune complexes, or influence the availability of the antigenic determinants in the Fc μ part of the IgM molecule, or again, it might fulfil both these functions.

3. Hydrophobicity

The increased precipitation obtained with IgM RF in the zone of high antigen in excess demonstrated the formation of more hydrophobic immune complexes. The complex-forming properties of certain IgG molecules have been related to the exposure of those hydrophobic amino acids that have aromatic residues (III, IV). The most pronounced increase in the amounts of precipitates was obtained for the rapidly sedimenting IgM RF which was associated with autologous IgG, whereas free 19S IgM RF molecules and rapidly sedimenting IgM without RF-activity gave more moderate increases in the amount of

precipitates. Thus it was obvious that rapidly sedimenting IgM components, irrespective of RF-activity, produced hydrophobic precipitates. When RF-activity occurred in a rapidly sedimenting IgM component, the hydrophobicity was more pronounced. There were indications that rapidly sedimenting IgM components without RF-activity contained albumin (M Uesson, unpublished observation, 1980), which would contribute to the hydrophobicity of such components.

The precipitin reaction in general is not fully understood. In most instances, and according to the lattice theory, it is obvious that at least three antigenic determinants per antigen would be necessary to obtain precipitation with divalent antibody molecules. An increased number of antigenic determinants would favour a more effective precipitation. However, the number of antigenic determinants are not always of crucial importance, as reported by Springer and Desai (1971) who found that haptens with a single determinant could precipitate with specific antibodies, whereas haptens with several determinants do not always precipitate (Boyd, 1973). According to Boyd's occlusion theory, hydrophobicity or decrease in polarity is more important to effective precipitation than is the number of antigenic determinants (Boyd, 1973). The various rabbit IgG anti-Fc μ used in this investigation (I) seemed to be directed against very general antigenic determinants in the Fc μ of IgM, as the demonstrated differences between IgM RF and normal IgM could be reproduced with any rabbit IgG anti-Fc μ antibodies.

In conclusion, this investigation demonstrated that precipitin reaction could be used to detect free as well as hidden IgM RF. The special deviation in the precipitin curve obtained for both molecules, compared to normal IgM, was related to the hydrophobicity of the molecules or complexes. The mechanisms behind the formation of these characteristic complexes included re-arrangement of immune complexes, multiple bonding within the complexes, and possibly conformational deviations of the IgM RF molecules.

Isolation and characterization of intermediate complexes and other components with common antigenic determinants (II)

When this work was begun, very few reports had appeared which described the isolation and characterization of intermediate complexes, and the number of patient sera was very limited. Moreover, the few cases reported showed differences in the mechanisms for complex formation. For example, intermediate IgG complexes containing abnormal 7S IgG, which at its Fc region combined with normal 7S IgG, were described in the serum of a patient with polyarthritis and the hyperviscosity syndrome (Heimer et al, 1971). The complex-forming properties of the abnormal IgG were not related to an anti-IgG activity. On the other hand, intermediate complexes isolated from three RA sera were formed by self-association of IgG RF molecules - i e, the RF-activity of the Fab γ part, and the antigenic determinants of the Fc γ part reactive with RF, were located on the same IgG molecule (Pope et al, 1975). It was not clear from these studies what proportion of the complexes was studied, nor whether any rearrangement of the complexes due to the choice of isolation method, or of ions for the isolation, had been taken into consideration. It soon became generally accepted that intermediate complexes in RA consist of self-associated IgG RF, which would mean that such complexes did not contain any autologous antigenic IgG that by other workers had been shown to react with RF (see rheumatoid factor activity). This was difficult to reconcile with the general view that RA was an auto-immune disease, in which autologous deviant IgG may act as immunogen or antigen.

In our study we wanted to isolate the total quantity of immune complexes from four sera, two of which exhibited RF-activity, while the other two contained hidden RF. The same method should be used for the isolation of complexes from all sera, to ensure identical treatment of the complexes, and that possible differences between the complexes were not due to different isolation procedures. Affinity chromatography to immobilized heat denatured human IgG was used, as it was

presumed that a minimum of one molecule within the complexes could have RF activity and affinity to the immobilized IgG. The complexes containing hidden RF would thus be rearranged due to the gel filtration during the isolation procedure (Bluestone et al, 1969). As eluants, we chose solvents capable of ensuring that molecules with high affinity to the ligand would be eluted. We found that the solvents at an acid pH commonly used in affinity chromatography to dissociate ligand-bound components did not fulfil our requirements. As was also reported by Dissanayake et al (1979), and as illustrated by some of the results reported in paper IV, molecules with a high affinity to the immobilized ligand, for example, were not eluted at an acid pH.

The isolated complexes, originating from the sera with hidden RF, exhibited RF-activity, which could be explained by a re-arrangement of the complexes during isolation. These complexes were formed at antigen excess as substantial amounts of the complexes were dissolved upon addition of normal IgG. The contrary was true for the complexes from the sero-positive sera, which increased in quantity after the addition of normal IgG, showing that these complexes were formed at antibody excess, at which RF had the capacity to bind more antigen. The presence of hidden RF thus occurred at antigen excess and when the patients were not diagnosed as RA, which may indicate that hidden RF is present at an early stage of the disease.

The isolated complexes were investigated as regards restrictions or deviations in comparison with the corresponding normal molecules in order to find systematic deviations which could be related to the complex formation. As discussed in the investigation, the complex formation was not solely related to self-association of identical molecules, although this could not be excluded as the mechanism of formation of some of the complexes. All complexes contained substantial amounts of Fab γ which lacked affinity to the immobilized IgG, indicating that some IgG RF were bound to other IgG molecules

lacking RF-activity. Three of the complexes contained mainly IgG of subgroup 1, while IgG3 predominated in the fourth.

IgG1 is a dominant subgroup in IgG RF (Shakib and Stanworth, 1978) as well as in intermediate complexes (Capra et al, 1971; Heimer et al, 1971), and both IgG1 and IgG3 occurred in the intermediate complexes isolated from a patient with the hyperviscosity syndrome (Gabriel and Hadler, 1981). We found, however, no indication that the subgroup of IgG had anything to do with the complex formation.

The content of sialic acid per mole of IgG in the isolated complexes was nearly four times higher than in normal IgG. This marked abnormality might be related to the complex formation, or to the physiological elimination of the complexes. It has been proposed that terminal sialic acid residues have a general role in determining the survival of glycoproteins in the circulation (Morell et al, 1971). Glycoprotein is removed from the circulation by the liver, after the terminal sialic acid has been removed enzymatically and the penultimate galactose exposed. This mechanism may be of importance for the removal of immune complexes from the circulation; and immune complexes containing desialylated orosomucoid as antigen have indeed been removed more rapidly from the circulation than complexes containing native orosomucoid, due to binding of the exposed galactose by cellular receptors in the liver (Finbloom et al, 1981). The existence of this mechanism has been further confirmed by results showing that the addition of covalently bound galactose to the antigen in small circulating immune complexes increased the elimination of such complexes by binding to liver hepatocytes (Rifai et al, 1982).

It is possible that the mechanism for removal of immune complexes dependent on the enzymatic removal of sialic acid will be impaired when the sialic acid content is excessive. The terminal galactose residues in the oligosaccharides of IgG may also be inaccessible to the galactose binding protein in hepatocyte membranes due to their location between the two C_H2 domains of the IgG Fc portion

(Silverton, 1977). Therefore, as an alternative to desialylation, it has been suggested that the galactose residues can also become exposed as a result of antigen induced conformational changes in IgG (Thornburg et al, 1980). The function of either of the two mechanisms for the elimination of immune complexes may be impaired by deviation - quantitative or qualitative - in the oligosaccharide composition, particularly by galactose or sialic acid deviation.

The relevance of such deviation in oligosaccharide composition to immune complex formation has been reported. An IgG with anti-globulin activity has been found to contain excessive amounts of both galactose and sialic acid in the $F(ab')_2\gamma$ part, the presence of sialic acid being related to the reactivity with the $Fc\gamma$ of normal IgG (Hymes et al, 1979). The presence of sialic acid on glycopeptides derived from certain mouse IgA antibodies has been correlated with the antibody activity, showing that the carbohydrate moiety of the antibody may influence the reaction with antigen (Matsuuchi et al, 1981). In another investigation, the galactose content of IgG from RA serum was found to be lower than normal, and probably related to instability of IgG and auto-immune reactivity (Mullinax et al, 1975). In the complexes studied here, however, we found no correlation between excessive sialic acid content and complex-forming properties, as desialylation had no observable effect on the complex formation (II).

We had found that the immune complexes contained IgG molecules with structural deviations in their carbohydrate content, and that the complexes possibly contained an autologous IgG of deviating conformation related to the complex formation. It was, therefore, of interest to investigate whether rabbit antibodies could be produced in response to immunization with isolated complex fractions. Unsuccessful attempts had earlier been made to produce rabbit antibodies against polyclonal IgG RF (Johnson et al, 1975), polyclonal IgG-dimers with RF-activity (Pope et al, 1974), and abnormal polyclonal IgG constituents of 13S complexes in hyperviscous human serum (Heimer et al, 1971).

Rabbits were immunized with the complex fractions isolated from one sero-positive and one sero-negative serum. After the antisera had been absorbed with normal human serum and with normal human IgG by affinity chromatography, antibodies were obtained which reacted specifically with components in the immune complex fractions. $F(ab')_2\gamma$ were derived from the antibodies and immobilized. Molecules with affinity to the immobilized ligands were present in all three sera analysed and there was also a good correlation in reactivity between the two affinity chromatography systems. It was of special interest to note that all three sera contained unique molecules, reactive with both antisera, thus indicating the existence of a certain general principle for the sera. Molecules with unique determinants were not related to RF-activity, as only a small amount of the components in the sera that had RF-activity contained the unique determinants. The unique molecules occurred in complexes in the sero-positive RA sera where RF was in antibody excess, whereas the unique molecules were 7S components in serum containing hidden RF and having an antigen excess of autologous IgG. Our results thus indicated that a 7S IgG in sero-negative RA serum contained the unique determinants, and that possibly such IgG was included in the complexes in the sero-positive RA sera and responsible for the presence of unique determinants in them.

It could not be excluded that a population of the rabbit antibodies reacted with IgM molecules, either due to shared determinants in IgG or IgM, or to the fact that one of the immunogenic complex preparations embodied rapidly sedimenting complexes containing IgM. Both of the immunogenic preparations contained immune complexes, and therefore the existence of antibodies directed against new determinants created by the complex formation (Nemazee and Sato, 1982) would have to be taken into consideration.

During the course of our investigation reports about the production of rabbit antibodies against heat-aggregated human IgG were published (Hunneyball and Stanworth, 1979; Arai and Shimizu, 1981).

In one case it was found that the antibodies were directed against a single antigenic determinant present in the Fc region of native human IgG, and it was proposed that the exposure of this determinant was potentiated after conformational changes due to heat-aggregation of the IgG (Hunneyball and Stanworth, 1979). In the other case, the antibodies were specifically directed against heat-aggregated human IgG, and it was also proposed that the antibodies possessed specific reactivity against conformationally altered IgG in immune complexes in a variety of diseases, - e g RA (Arai and Shimizu, 1981), although no further specification of the antibody reactivity was made. We used the isolated $F(ab')_2\gamma$ of the specific rabbit IgG antibodies, but Arai and Shimizu (1981) used intact molecules, which raises the possibility that their antibody reactivity with immune complexes from individuals with RA was partly due to RF reactivity with the rabbit IgG.

Rabbit antibodies have been raised against idiotypic and cross-idiotypic determinants in polyclonal IgM RF, monoclonal IgM RF, and monoclonal IgG RF (see rheumatoid factor structure), but there are no reports of such determinants in polyclonal IgG RF. The occurrence of our unique determinants was not related to the RF-activity in general, implying that the cross-idiotypic determinants, if present, would have reflected the conformation of only a limited population of idiotypic RF molecules.

Circular dichroism of immune complexes with rheumatoid factor activity (III)

This investigation was prompted by earlier results showing that RF-activity was not a property of all IgG molecules in IgG complexes in RA serum, and that immune complexes containing RF also contained molecules with unique determinants (II). There were thus indications that some IgG molecules in the immune complexes functioned as antigen and other indications that a population of the IgG molecules were deviant in structure. The purpose now was to investigate whether the presence of a presumed deviant structure of IgG in such

immune complexes could be verified by circular dichroism (CD) analysis.

Several indications that such IgG existed in RA serum had been reported. It had been shown that the fragments obtained after papain hydrolysis of IgG from RA serum were different from those of normal IgG (Watkins et al, 1970), indicating that the conformation of the RA IgG was different from that of normal IgG. Based on studies of the catabolism of rheumatoid and normal IgG injected into mice, Watkins et al (1971) proposed that the pool of rheumatoid IgG was more immunogenic than normal IgG, which indicates structural anomaly in rheumatoid IgG. It was suggested that the deviant structure of rheumatoid IgG caused increased catabolism of autologous IgG in RA patients compared to normal individuals (Watkins and Swannell, 1973). Deviations in the CD spectrum of the pool of rheumatoid IgG, compared to normal IgG (Johnson et al, 1974 a), later led to the proposal that RF is produced against conformationally altered IgG in RA (Johnson et al, 1975).

In this investigation we used IgG complexes isolated from RA serum. As reported earlier (II), in addition to RF the complexes contained IgG molecules without RF-activity, and a minor population of the complexes contained molecules with unique determinants, which also occurred in other RA serum (II). The first question was whether the conformation of the IgG constituent of the complexes was deviant, compared with that of non-complexfoming IgG isolated from the same RA serum, and with that of normal pooled IgG. The second question was whether a presumed deviant conformation of the molecules in the population of complexes with unique determinants could be verified by a deviant CD spectrum.

CD measurements were made at pH 7.3 when the complexes were intact, and at pH 5.4 and pH 11.0 after they had dissociated. The slightly acid solution at pH 5.4 had no observable denaturing effect on normal IgG, and no irreversible denaturation of normal IgG

occurred at pH 11.0, which was in agreement with results reported by Dorrington and Smith (1972).

By CD analysis, it was shown that the conformation of the IgG obtained by dissociation of the IgG complexes differed from that of the non-complexforming IgG from the same individual. Compared with non-complexforming IgG and normal IgG, which had identical CD spectra, the CD spectra obtained at pH 5.4 displayed stronger absorption bands in those parts of the near ultraviolet region attributed to tryptophan, other aromatic amino acids, and disulphide bonds in asymmetric environments for the IgG monomers obtained from the complexes. Since IgG1 should generally exhibit the same CD as normal IgG (Johnson et al, 1974 b), the deviant CD spectrum was unrelated to the restriction of the complex-forming IgG to subclass 1; thus it could only be related to the complex-forming properties of the IgG, indicating that these properties in turn were related to the exposure of the aromatic residues of certain hydrophobic amino acids.

The optical activity of tryptophan was further increased by the complex formation per se, as the positive ellipticity at 295 nm was more pronounced at pH 7.3 when the complexes were intact, than at pH 5.4 after dissociation. Conformational changes in the Fc part of antibody, after its Fab part has reacted with antigen, can be expressed by changes in the circular polarization of the fluorescence emitted by tryptophan residues (Schlessinger et al, 1975). By CD analysis it has been shown that such conformational changes are expressed by an increased positive ellipticity at 290.5 nm, which is also attributed to the tryptophan chromophores (Forster and Sela, 1979). This indicated that the more pronounced CD at about 290 nm obtained for the intact complexes could be related to conformational changes in the Fc γ part upon complex formation.

The CD was determined for intact complexes with unique determinants at pH 7.3 and at pH 11.0 after the complexes had dissociated. Since the components with unique determinants included mainly IgG, but

also IgM and IgA, the CD spectra had to be compared with the theoretical CD, calculated for a mixture of the corresponding normal proteins at corresponding ratios. This could explain why the CD deviations earlier observed for complex-forming IgG in the near ultraviolet region were not observed when comparing the CD of molecules with unique determinants and the CD of the reference mixtures. The CD deviations obtained for molecules with unique determinants were mainly expressed by an increased negative ellipticity at 218 nm, compared to a reference CD spectrum, both for the intact complexes and the monomeric constituents, and also by an increased positive ellipticity for the monomers at 254 nm. It had previously been shown (II) that the unique determinants not only occurred on immune complexes, but also on monomeric IgG molecules. It was therefore of interest to note that the CD deviations observed for the complexes with unique determinants were also valid for the monomeric constituents.

The positive band at 254 nm appeared at pH 11 for all IgG studied. The appearance of this band is pH-dependent and related to denaturation of the IgG, which under these conditions is completely reversible according to CD analysis as shown by Dorrington and Smith (1972), who suggested that the band was due to ionization of the phenol moiety of tyrosine residues. The positive ellipticity obtained for immunoglobulin monomers with unique determinants was almost twice that of the reference mixture, indicating an increased optical activity of tyrosine residues in the unique monomers.

The 30 % increase in the negative ellipticity at 218 nm exhibited by monomers with unique determinants could be explained by deviations in the secondary structure. Deviations of a similar magnitude at 218 nm have been reported for individual purified rabbit antibodies, compared with normal rabbit IgG (Bear et al, 1974), thus linking the deviation to antibody specificity. The monomers with unique determinants might also comprise a population with special antibody properties since the determinants were localized to the $F(ab')_2\gamma$ part

(II). The band at 218 nm is characteristic of IgG and has been attributed to the β -structure or to contributions from side chain transitions (Dorrington and Smith, 1972). The β -structure is recurrent in both the V and the C domains of the IgG molecule (Marquart and Deisenhofer, 1982). The deviating CD at 218 nm might thus be related to the secondary conformation in the variable part of the monomer immunoglobulins with unique determinants or, as suggested by Bear et al (1974) to differences in hydrogen bond positions and orientations, not necessarily confined to the β -sheet structure.

Circular dichroism of immune complexes, IgG and Fab γ with unique antigenic determinants from rheumatoid serum (IV)

At the outset of this investigation we already knew that unique determinants occurred in a minor population of IgG monomers, intermediate and rapidly sedimenting complexes in rheumatoid serum (II). The unique determinants had been located to the F(ab')₂ γ region of IgG in one case (II) and there were indications from CD analysis that the conformation, of the variable part of the monomers with unique determinants, might also deviate from the normal (III). The complex-forming IgG in general had a deviating CD spectrum compared with that of non-complexforming IgG and with that of those molecules containing unique determinants (III). As the latter molecules constituted both IgG and IgM, CD deviations in this case could not be related specifically to the IgG structure. Neither could the CD spectrum of complex-forming IgG in general be related to the CD spectrum of the immunoglobulins with unique determinants, as the proportion of unique molecules in the fraction containing complex-forming IgG was not known. Furthermore, although there were indications, it had not been experimentally verified that the unique determinants resided in the variable region also of complex-forming immunoglobulins.

The purpose of this investigation was to clarify these points. IgG-IgG and IgG-IgM complexes with unique determinants were therefore

isolated from RA serum by the use of a third specific rabbit anti-serum not previously used. The isolated complexes were dissociated, the presence of the unique determinants in the isolated IgG was investigated and the conformation of IgG with unique determinants was analysed by CD. Finally, the presence of unique determinants in the Fab γ obtained from IgG with unique determinants was controlled and related to the conformation analysed by CD.

The monomeric IgG, obtained by dissociation of the complexes with unique determinants at pH 11, formed complexes again at a neutral pH, and all of these newly formed IgG-IgG complexes exhibited unique determinants. It could now be demonstrated that the IgG complexes with unique determinants had a CD spectrum similar to that earlier obtained from the same rheumatoid serum (III) for complex-forming IgG in general. The abnormal CD spectrum earlier obtained (III) could now thus be related to the presence of unique determinants in the IgG molecules, and the unique determinants could in turn be related to the special IgG conformation that exhibited an increased optical activity of aromatic amino acid residues (III). Both the presence of the unique determinants, and the exposure of certain hydrophobic amino acid residues were thus related to the complex-forming properties of the IgG molecules.

When the IgG complexes with unique determinants were dissociated in a solvent containing glycine-HCl at pH 3, the CD spectrum was "normalized" and similar to that of normal IgG. This was not caused by the dissociation of the complexes per se, as the special conformation had earlier been shown to be an inherent property of the complex-forming IgG monomers (III). It was therefore presumed that the special IgG conformation was "normalized" in the acidic and polar glycine solvent. The question then was whether the expression of the unique determinants was entirely correlated with the CD spectrum - i.e., was the "normalized" CD spectrum correlated with a functional absence of unique determinants? This was investigated by controlling whether the molecules had affinity to the immobilized F(ab') $_2\gamma$ of the

specific rabbit IgG antibodies also in glycine-HCl at pH 3. We found that about one third of the molecules exhibited unique determinants also at pH 3 in the solvent containing glycine-HCl, and that this subpopulation of immunoglobulins had a unique CD spectrum at the same pH.

The isolated IgG complexes with unique determinants contained two populations of IgG molecules, only one of which with unique determinants. This was confirmed when the proportions of Fab γ fragments with unique determinants was determined. The same proportion (one third) of Fab γ had unique determinants at neutral pH as earlier observed for the subpopulation of immunoglobulins exhibiting unique determinants at pH 3. The isolated Fc γ fragments contained no unique determinants. The deviations demonstrated in CD for Fab γ fragments with unique determinants at neutral pH, and for those immunoglobulins exhibiting unique determinants at pH 3, were similar, and mainly reflected the optical activity of the aromatic amino acid residues. It was thus obvious that Fab γ with unique determinants and a deviating exposure of certain hydrophobic amino acid residues were included in the immune complexes, and that these conformations were correlated to the complex formation.

One question of great relevance is whether unique determinants occur in RA serum in general. It has been demonstrated in a blind investigation of eleven sera from individuals with various diseases of the joints, of which six had RA, that the occurrence of immunoglobulins with unique determinants here reported (IV) is associated with RA (U-B Hansson, unpublished observation, 1983).

One possibility was that the unique determinants were located in the Fab γ of an anti-idiotypic IgG molecule, which had antibody specificity directed against idiotypic determinants in the Fab γ of IgG RF. Anti-idiotypic antibodies may have an internal image of the antigen, against which the idiotypic antibody is directed (Nisonoff and Lamoyi, 1981). Our unique determinants, if located in the Fab γ of anti-

idiotypic IgG could thus be similar to those of the antigenic or immunogenic IgG, against which the presumed idiotypic IgG RF was directed. We had some experimental evidence that this model was possible, as the Fab γ with unique determinants could react with the Fab γ without unique determinants, derived from the same IgG complexes (U-B Hansson, unpublished observation, 1982). This reaction might occur physiologically concomitant with that between IgG RF and the Fc γ part of the IgG antigen. In such a model, three species of IgG would be present in the complexes: anti-idiotypic IgG (directed against Fab γ of idiotypic RF), idiotypic IgG (RF directed against Fc γ of autologous IgG), and autologous IgG (the antigen for RF). This model and the reactions described were most likely, however, oversimplifications of the problem. For example, RF could probably also react with the Fc γ part of the anti-idiotypic IgG, and possibly even with the Fc γ part of at least some other IgG RF molecules (II; Pope et al, 1975).

As a further complication of this model, it should be noted that small amounts (about 5 %) of human polyclonal Fab γ contain determinants which possess Fc γ -like properties, as regards reactivity with protein A (Biguzzi, 1982). These Fc γ -like determinants were probably located in the framework conformation of the V domains of the Fab γ , showing that subsets of immunoglobulin molecules may possibly have both effector functions and antigen recognition in the variable regions. The presence of Fc γ -like determinants was demonstrated by reaction with protein A (Biguzzi, 1982) whose reaction with the Fc γ is known to be mediated by a hydrophobic contact within the junction of the C $_H$ 2-C $_H$ 3 domains (Deisenhofer et al, 1978). Thus, those determinants that were general for Fab γ and Fc γ were hydrophobic in character. It is known that RF can react with determinants in the C $_H$ 2 and C $_H$ 3 domains of Fc γ , or in both (Johnson and Faulk, 1976). It is possible that these determinants are also of a hydrophobic character, as RF reacts better with heat-denatured IgG (which should be more hydrophobic) than with native IgG (Gaarder and Michaelsen, 1974). Thus, the model could be complicated also by reactions between IgG RF and

Fab γ of "general" IgG in the complexes. The physiological effect of these theoretically possible interactions would most probably be a stabilization of the complexes, due to multiple bonds between only a limited number of different populations of IgG molecules.

CONCLUDING REMARKS

Hidden IgM RF in strong association with autologous antigen and exhibiting no RF-activity can now be detected. The new principle in the method described is related to hydrophobicity. The mechanisms behind the increased hydrophobicity are related to secondary immune reactions influenced by IgM RF, such as re-arrangements of immune complexes and multiple bonding within immune complexes. Possibly conformational deviations of the IgM RF are also involved. The new principle might also be applied to detect IgG RF and immune complexes containing IgG RF. The mechanisms demonstrated here might be used to develop new immunochemical methods for routine analysis.

The existence of unique markers on IgG molecules in RA serum has now been verified. These markers can be employed for the isolation and characterization of unique IgG molecules. The IgG molecules are involved in the formation of immune complexes in RA serum, and can be useful instruments for further investigation aimed at elucidating the mechanisms behind the formation of immune complexes in RA.

FUTURE PROSPECTS

It is clear that rheumatoid factor, immune complexes, and a possible antigenic molecule, (either IgG, or one structurally related to IgG), all play a role in the immunopathology of rheumatoid diseases.

The present pharmacotherapy of rheumatoid diseases is mainly directed at an alleviation of pain and inflammation, which are the major symptoms. This can be achieved by the administration of derivatives of salicylic acid, non-steroidal anti-inflammatory drugs and glucocorticoids. A certain remission of disease activity can be achieved by administration of kinoline derivatives, D-penicillamine or gold salts; when no remission is obtained with any of these, cytostatic drugs are used to obtain a general immunosuppression.

Apart from the pharmacotherapy, extracorporeal treatment of the blood can be achieved by plasmapheresis, which is a method for removal of whole blood plasma. This treatment results in a non-specific decrease of all plasma proteins, including the deleterious ones.

Active and specific therapy for rheumatoid diseases would require new means to reduce or eliminate specifically those immunoglobulins thought to be involved in the immunopathogenesis of the diseases. A few ideas about how biological molecules could theoretically be used are proposed:

The use of anti-idiotypic monoclonal antibody in vivo in the treatment of auto-immune disease has been suggested by McMichael and Bastin (1980). Anti-idiotypic antibodies, directed against idiotype RF, could be raised in animals, or by the generation of monoclonal antibodies from hybridomas. Such antibodies could be immobilized to a suitable matrix for extracorporeal circulation of the blood and removal of the RF molecules. Possibly anti-idiotypic antisera could be used in vivo to interact directly with the immunoglobulin producing plasma cells, but

as stated by Førre et al (1979): "Before anti-idiotypic antisera can be used to attack and destroy RF-producing cells and thus possibly be used as a very specific therapeutic instrument in rheumatoid arthritis, more work is needed to define the final specificities of the RF". One problem is that many different RF idiotypes exist, only some of which are cross-idiotypic and exist in several homologous sera, which precludes the use of this method on a general basis. Possibly the method, if economically feasible, might be effective for the treatment of individual patients.

It has been suggested that antibody affinity is an important factor in the production of injurious antigen-antibody complexes (Steward, 1981), and as proposed by the author: "Should the association of low affinity antibody production with chronic disease be confirmed, consideration could then be given to possible immunomodulation aimed at enhancing antibody affinity as a means of treating chronic antigen-antibody complex disease". We have shown the possible existence of autologous human anti-idiotypic antibodies, involved in the immune complex formation in RA serum (IV). It has been shown that anti-idiotypic antibodies prepared against idiotype anti-insulin antibodies can interact with insulin receptors on tissues and thus physiologically mimic the action of insulin itself (Sege and Peterson, 1978). The possibility of utilizing the so-called internal image of the antigen in anti-idiotypic antibodies for vaccine production has been raised (Nisonoff and Lamoyi, 1981). As pointed out by Roitt et al (1981), who have described a general strategy for the manipulation of the immune response with monoclonal anti-idiotypic, it may be necessary to combine the administration of anti-idiotypic with other manipulations such as immunosuppressive drugs or removal of circulating idiotype from serum, in order to obtain the desired effect. Thus, possible anti-idiotypic antibodies with relevance for immune complex formation could be isolated (IV) from rheumatoid serum and be used together with a potent adjuvant for immunization of the patient. If the anti-idiotypic antibodies contained an internal image of the antigen, sufficiently immunogenic to stimulate the synthesis of

high-affinity, "normal IgM and IgG antibodies", there should be a theoretical possibility of these newly produced antibodies reacting not only with the "internal image", but also with the physiological antigen for RF and eliminating or decreasing the content of this harmful molecule.

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Quantitative Precipitin Analysis of Free and Hidden RF IgM

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Uesson, M. & Hansson, U.-B. Quantitative Precipitin Analysis of Free and Hidden RF IgM. *Scand. J. Immunol.* 14, 61–69, 1981.

Quantitative precipitin analysis using rabbit IgG anti-human $\text{Fc}\mu$ was performed with 16 RF IgM, six macroglobulinaemic IgM and four normal IgM. Abnormal precipitin curves were obtained for all RF IgM, even when the latter were not readily demonstrated with conventional serological tests for RF, and for macroglobulinaemic IgM with sedimentation rates greater than 19S. These IgM formed significantly more precipitates with IgG anti- $\text{Fc}\mu$ in the antigen excess zone than did normal IgM, but the precipitin curves for the other zones were similar for all IgM. The underlying mechanisms of some of the reactions were studied and discussed. Because the divergence in the precipitin reaction for normal IgM and RF IgM was so pronounced, a model precipitin curve was constructed. This could be used to detect RF IgM, even when not readily demonstrable with conventional serological tests for RF, by direct analysis of serum. The results obtained for RF IgM suggested that the method might be applied to RF IgG and intermediate complexes comprised of IgG. The mechanisms demonstrated here might be used to develop immunological methods for routine use.

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There is a need for a method capable of detecting all rheumatoid factor IgM (RF IgM) and that functions whether an anti-IgG activity of RF can be demonstrated or not. A combination of the following data suggests how such a method could be developed. RF IgM and normal IgM give precipitin curves with anti-IgM that differ so that the precipitation especially in the antigen excess zone is higher for RF IgM than for normal IgM [19]. A decreased solubility in the antigen excess zone on quantitative precipitin analysis has been ascribed to available determinants in the antigen [11], and molecular weight of the antigen [13, 16, 20, 21], and to impurities in the antigenic preparation [6].

RF IgM with detectable anti-IgG activity would thus give a deviating precipitin curve because it can react with $\text{Fc}\gamma$ of rabbit or human IgG [12] or antigen–antibody complexes [1, 10]. The precipitin reaction might also be influenced by the IgM in the RF IgM–IgG complexes having another conformation or by the complexes being larger than normal IgM. Strongly

hidden RF, saturated with antigen, would produce differences owing to their abnormal conformation or size.

In the present work, quantitative precipitin analysis using rabbit IgG anti-human $\text{Fc}\mu$ was performed with 16 RF IgM, 6 macroglobulinaemic IgM and 4 normal IgM. Not only RF IgM with anti-IgG activity but also hidden RF IgM and rapidly sedimenting IgM (greater than 19S) were detected. The results indicated that the principles used here for detecting RF IgM could be used also for detecting RF IgG and intermediate complexes comprised of IgG.

MATERIALS AND METHODS

Sera. Twenty-two sera from patients with rheumatoid arthritis (RA) were used. Fifteen of these sera were analysed separately and seven (R11–R17) were pooled (RA serum pool) before analysis.

Six sera (M1–M6) lacking rheumatoid factor (RF) activity, from patients with Waldenström's macroglobulinaemia, were used.

Twelve normal sera (N1–N12) lacking RF activity were collected from laboratory personnel. N1 and N2 were used separately and N2–N8 and N9–N12 were used as separate normal serum pools.

Most sera were stored at -20°C before use.

Antiserum to human $\text{Fc}\mu$. Seven pools of rabbit antiserum to human $\text{Fc}\mu$ were prepared. 19S IgM isolated (see below) from sera of patients with Waldenström's macroglobulinaemia was used as immunogen. One milligram of purified macroglobulinaemic 19S IgM dissolved in 0.5 ml 0.16 M NaCl and mixed with 0.5 ml complete Freund's adjuvant (Difco, Detroit, Mich., USA) was injected to several sites behind the scapulae of each rabbit. The rabbits were given booster doses in the same manner when required. Each of the seven pools of anti- $\text{Fc}\mu$ was obtained from several bleedings of two or more rabbits.

The specificity of the antisera was assessed by double immunodiffusion and immunoelectrophoresis. Antibodies against undesired serum proteins were removed by the addition of the appropriate isolated serum proteins directly to the antiserum or by affinity chromatography using these proteins coupled [7] to Sepharose CL-4B. All antisera were rendered specific for $\text{Fc}\mu$. This was checked by double immunodiffusion against isolated kappa (κ) and lambda (λ) light chains, $\text{Fab}\mu$, $\text{Fc}\mu$ and intact 19S IgM or by immunoelectrophoresis against macroglobulinaemic 19S IgM digested with papain. The papain digestion of IgM and the preparation of $\text{Fab}\mu$ and $\text{Fc}\mu$ fragments were done as described by Mihaesco & Seligmann [25]. A single precipitin arc should be produced against $\text{Fc}\mu$ and 19S IgM on double immunodiffusion and against the anodal $\text{Fc}\mu$ component of the papain-digested IgM on immunoelectrophoresis.

IgG was prepared from the antisera by salting out in 1.84 M $(\text{NH}_4)_2\text{SO}_4$ overnight at 4°C or by ion-exchange chromatography on a column of DEAE-Sephacel, from which the IgG was eluted with 0.02 M sodium phosphate, pH 8.0.

$\text{F(ab')}_{2\gamma}$ anti- $\text{Fc}\mu$ was isolated from one IgG anti- $\text{Fc}\mu$. The IgG was pepsin-digested [32], applied to a column of Sephacryl S-200, and eluted with 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0. The appropriate components were selected. The absence of undigested 7S IgG was checked by analytical ultracentrifugation against a standard human 7S IgG preparation and by immunoelectrophoresis. In these experiments the protein concentration in the $\text{F(ab')}_{2\gamma}$ anti- $\text{Fc}\mu$ fraction was 10 g/l, and control experiments showed that 2% IgG in the samples would be detected.

Titre determinations of IgG anti- $\text{Fc}\mu$ were made as described by Becker [4] by single radial immunodiffusion (SRI). (See quantitation of IgM for details.) The SRI titres for the various anti- $\text{Fc}\mu$ varied between 190 and 2045 μg IgM per ml antiserum.

Gel filtration of RA serum. Gel filtration of RA serum was performed on Sephadex G-200. The samples R18 and R19 were treated with EDTA, NaCl and glycine as described previously [17] before elution was performed in 0.75 M phosphate-buffered

saline, pH 7.35 [5]. The other sera were eluted with 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0. All IgM fractions then obtained showed RF activity.

Isolation of IgM. Macroglobulinaemic IgM was precipitated as euglobulin or in 2.5 M $(\text{NH}_4)_2\text{SO}_4$ at pH 7.0. The dissolved precipitates were recycled on Sephadex G-200 in 0.05 M sodium phosphate, 0.5 M NaCl at pH 7.0, and the isolated IgM fractions were further purified by preparative electrophoresis in 1% agarose gel at pH 8.6. The purity of IgM was assessed by immunoelectrophoresis, double immunodiffusion and polyacrylamide electrophoresis. The IgM concentration was calculated from the light absorption at 280 nm ($E_{1\%}^{1\text{cm}}$ 280 nm of 12.5) [24].

Isolation of normal rabbit IgG. IgG from non-immunized rabbits was prepared from serum by salting out in 1.84 M $(\text{NH}_4)_2\text{SO}_4$ overnight at 4°C . The washed precipitates were dissolved and dialysed against 0.16 M NaCl. The preparation was then applied to a column of DEAE-Sephacel from which the IgG was eluted with 0.02 M sodium phosphate, pH 8.0. The purity of the IgG was checked by analytical ultracentrifugation against a standard human 7S IgG preparation.

Quantitative precipitin reaction. Appropriate amounts of isolated IgM or IgG in whole serum were added to an adjusted and constant volume of IgG anti- $\text{Fc}\mu$ corresponding to 0.2 ml of anti- $\text{Fc}\mu$ with the SRI titre of 865 μg IgM per ml antiserum and diluted with 0.16 M NaCl to a total volume of 2.2 ml. The precipitates formed after 48 h at 4°C were centrifuged down (3400 g, 10 min), washed three times with cold 0.16 M NaCl and dissolved in 1 ml 2 M NaOH. The absorbance was measured at 280 nm ($E_{1\%}^{1\text{cm}}$ 280 nm of 12.5) [24]. The amount of IgM (range 0.08–1.10 mg) added (abscissa) was plotted against total mg protein precipitate (ordinate). (See Figs. 1–4.)

All analyses except those with R3 and R4 were made in duplicate and in all of them the maximum deviation from the mean was 0–17.7% (median value 3.8%) for the analyses with 0.08 mg IgM; 0–9.8% (median value 1.9%) for the analyses with 0.4 mg IgM; and 0–12.2% (median value 2.2%) for the analyses with 0.6 mg IgM.

Optimal antigen to antibody ratios were estimated by gel diffusion [28].

Fc-mediated immune precipitation of antigen-antibody complexes [27] was observed when using isomolar concentrations of IgG anti-human serum albumin (HSA) and the corresponding $\text{F(ab')}_{2\gamma}$ anti-HSA in quantitative precipitin analysis of HSA. Up to 50% of the antigen was precipitated in the antigen excess zone due to Fc-mediated immune precipitation.

When the precipitin reaction was performed in this study with $\text{F(ab')}_{2\gamma}$ anti- $\text{Fc}\mu$, a 30% molar excess over the corresponding intact 7S IgG was used to obtain the same amount of precipitate with 0.4 mg IgM. Thus, any Fc-mediated precipitation would remain concealed in our experiments.

The same solvents, volumes, reaction times, and temperatures were used in all of the quantitative precipitin analyses. The criteria to be considered when different quantitative precipitin reactions are

compared, as discussed by Day [9], were thus taken into account.

Quantitation of IgM. Quantitation of IgM was made on the reduced (0.1 M β -mercaptoethanol, pH 7.0, 4°C, overnight) samples by single immunodiffusion against rabbit IgG anti-Fc μ . RF IgM can release antigen in antigen-antibody complexes [2]. Any errors due to this effect were avoided by the use of reduced IgM. A standard human serum preparation (Behringwerke AG, BRD) was used as IgM reference, and a standard curve was constructed for each immunoplate. Duplicate analyses were made with two or more dilutions of each samples. The maximum deviation from the mean was 0.4–17.8% (median value 2.9%).

Ultracentrifugal analysis. Ultracentrifugal analysis was done with an An-D rotor at 59,780 rev/min (259,700 *g*) in a Beckman Analytical Ultracentrifuge Model E with Schlieren optics at 20°C. The total protein concentration was below 20 g/l, and no correction was made for the Johnston-Ogston effect. $s_{20,w}$ values were obtained by making the usual correction for density and viscosity using a $\bar{v}$ value of 0.723 [26]. The $s_{20,w}^0$ [14] and the sedimentation-rate distribution of the components in the samples [15] were determined as described previously.

Detection of rheumatoid factor activity. Rheumatoid factor activity was detected with two methods: (1) Waaler-Rose test to detect free RF. Complement was inactivated by heating serum at 56°C for 30 min. The haemagglutination test was carried out as described by Winblad [33]. The test was considered positive when the titre was 64 or more. Rabbit IgG anti-sheep erythrocytes were used to coat the erythrocytes. (2) Method to detect hidden RF [17]. The same erythrocytes and rabbit IgG anti-sheep erythrocytes were used as in the standard method. Complement was inactivated by adding 0.1 ml 0.002 M EDTA, pH 7.0, to 0.5 ml serum and leaving it at room temperature for 2 h. The EDTA-serum mixture was then made 1 M with respect to NaCl and kept at room temperature over-night. Next, the haemagglutination test was performed. A solution of 0.01 M glycine in 0.16 M NaCl, pH 7.2, was used as diluent in the titration. Titres were compared with those obtained by the standard method. Titres were not corrected for the 20% dilution due to addition of EDTA.

Electrophoretic and immunodiffusion methods. Agarose (1%) gel electrophoresis was run in 0.075 M barbital buffer made 2 mM with regard to calcium lactate at pH 8.6. Immunoelectrophoresis was run in 1% agarose gel in accordance with the method of Scheidegger [30]. Polyacrylamide (T=3.6%, C=0.09%) gel electrophoresis was run by the method of Davis [8], double immunodiffusion by the method of Ouchterlony [29] and single radial immunodiffusion by the method of Mancini *et al.* [22].

RESULTS

Figure 1 gives a typical precipitin curve obtained in this study. We present in the rest of this work

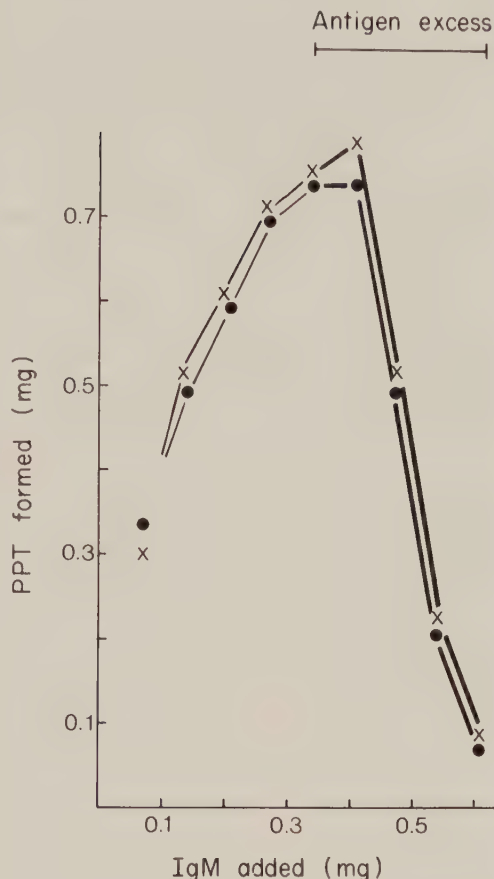


FIG. 1. Curves obtained on quantitative precipitation of IgM (M2). The experimental values from analyses on two different occasions are given. Total amount of precipitates (ordinate) obtained on reaction of a constant amount of IgG anti-Fc μ with various amounts of IgM (abscissa).

mainly the antigen concentrations that were of special interest, namely 0.08 mg IgM (antibody excess), 0.4 mg IgM (maximum; in slight antigen excess) and 0.6 mg IgM (in higher excess of antigen.)

The quantitative precipitin reactions of a pool of sera from patients with seropositive rheumatoid arthritis (RA serum pool) and a pool of normal sera as a control with IgG anti-Fc μ were studied (Fig. 2). Our objective was to compare the precipitin curves in the three zones of interest. The RA serum pool gave considerably larger amounts of precipitates in the zone of higher antigen excess than the normal serum pool. No significant differences in amount of

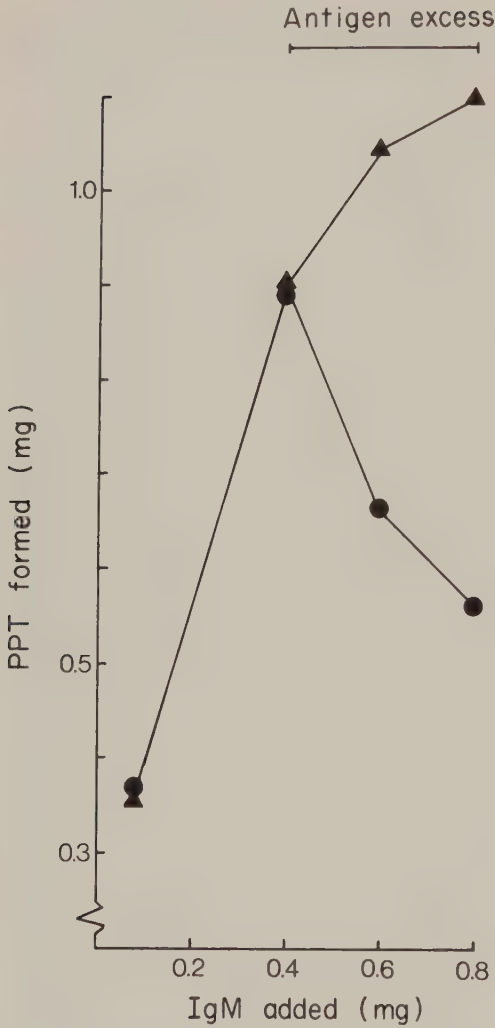


FIG. 2. Curves obtained on quantitative precipitation of a normal serum pool (●) and an RA serum pool (▲). Total amount of precipitates (ordinate) obtained on reaction of a constant amount of IgG anti- $Fc\mu$ with various amounts of antigen (abscissa).

precipitates could be observed in the regions of antibody or slight antigen excess.

The precipitin reaction was then determined for one normal and two individual RA sera. These individual samples gave precipitin curves that were similar to those obtained with the above-mentioned serum pools. The amount of IgM in the supernatants corresponding to 0.6 mg IgM was determined (Table I). The same amount of IgM was found in all three supernatants. The differences in amount of precipit-

ates (Table I) could not be explained by differences in amounts of IgM in the precipitates.

TABLE I. Quantitative precipitation of 0.6 mg IgM in two RA sera and one normal serum with rabbit IgG anti- $Fc\mu$: amount of precipitates obtained and amount of IgM in supernatants

Sample	Total precipitate (mg)	IgM in supernatant (mg)
R3	0.60	0.38
R4	1.65	0.36
N2	0.05	0.36

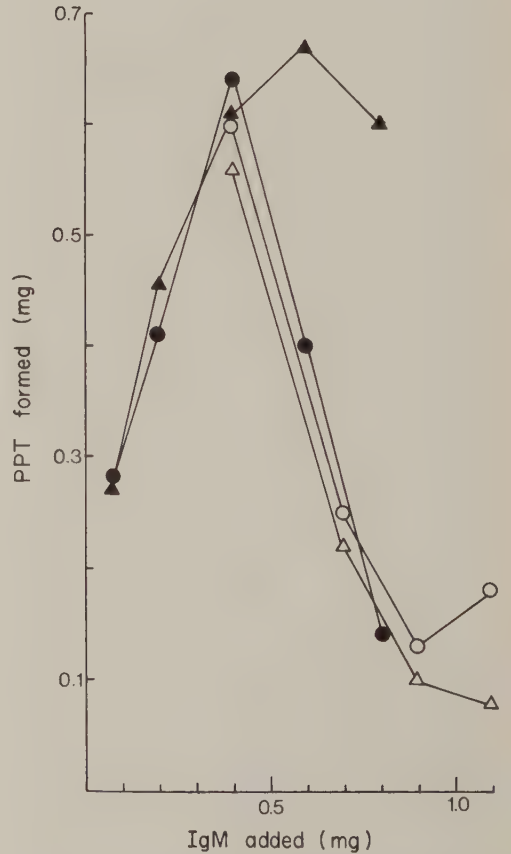


FIG. 3. Curves obtained on quantitative precipitation of R22 (▲) and N9-N12 (●) using IgG anti- $Fc\mu$ and the corresponding curves obtained of R22 (△) and N9-N12 (○) using $F(ab')_2\gamma$ anti- $Fc\mu$. Total amount of precipitates (ordinate) obtained on reaction of a constant amount of IgG anti- $Fc\mu$ or $F(ab')_2\gamma$ anti- $Fc\mu$ with various amounts of antigen (abscissa).

The precipitin reactions of one RA serum and one normal serum pool with intact IgG anti-Fc μ and its isolated F(ab')₂ γ fragments were then compared (Fig. 3). The precipitation with intact IgG anti-Fc μ differed for the two samples in the zone of higher antigen excess, but with the F(ab')₂ γ anti-Fc μ it was nearly identical.

The aim of the next experiment was to find out whether the differences observed in the reaction of RF IgM with IgG anti-Fc μ and its F(ab')₂ γ fragments depended on a reaction of RF IgM with normal rabbit IgG. IgG from non-immunized rabbits, in the same amount as used earlier for IgG anti-Fc μ , was therefore added to the supernatants from the quantitative precipitin analysis with the F(ab')₂ γ anti-Fc μ shown in Fig. 3. The same maximum amount of precipitate (0.3 mg) was formed in the tubes, corresponding to 0.7 mg IgM for the RA and the non-RA sample. That both samples contained IgM in excess after precipitation with normal rabbit IgG was shown by addition of intact IgG anti-Fc μ to the supernatants. The same maximum amount of precipitates (0.7 mg) was formed in the tubes, corresponding to 0.7 mg IgM for both samples. The precipitin reaction of RF IgM with IgG anti-Fc μ could thus not be explained by the assumption that RF IgM could precipitate more rabbit IgG than normal IgM.

To ascertain whether the precipitin reaction with IgG anti-Fc μ was influenced by the *s* value of IgM, quantitative precipitin analysis was performed with six isolated macroglobulinaemic IgM that had different *s* values but no RF activity (Table II). The IgM with a higher *s* value than normal IgM gave the same precipitin reaction as earlier observed for RF IgM, whereas 19S IgM gave the same precipitin reaction as normal IgM.

TABLE II. Quantitative precipitation of macroglobulinaemic IgM with rabbit IgG anti-Fc μ : regression coefficients (*K*) for the line between the two points of precipitates at 0.4 and 0.6 mg IgM obtained for normal (19S) and rapidly sedimenting (greater than 19S) IgM

Sample	<i>s</i> _{20w} ⁰	<i>K</i>
M1	19	-2.3
M2	19	-3.3
M3	19	-2.5
M6	19	-2.5
M4	20	0.4
M5	21	0.3

The results obtained on quantitative precipitin analysis of 11 individual and one pool of seropositive RA sera with rapidly sedimenting (greater than 19S) or 19S RF IgM are given in Table III. The regression coefficients for the line between the two points of precipitates at 0.4 mg IgM and 0.6 mg IgM were positive in all cases except two. The samples containing the rapidly sedimenting IgM and the RA serum pool showed significantly higher regression coefficients than the individual sera that contained only 19S IgM. The increase in amount of precipitates obtained for 0.6 mg IgM compared with for 0.4 mg IgM was thus largest for samples that contained rapidly sedimenting components or were a pool of several RA sera.

TABLE III. Quantitative precipitation of RA sera containing RF IgM with rabbit IgG anti-Fc μ : regression coefficients (*K*; see Table II) obtained for RF IgM with different *s* values and RF titres

	Sample	RF titre*	<i>s</i> value	<i>K</i>
Rapidly sedimenting				
High RF titre	R1	1024	27	0.7
	R2	2048	26	0.4
Low RF titre	R4	64	22	2.1
	R5	512	22	0.8
Normally sedimenting				
High RF titre	R22	1024	19	0.3
Low RF titre	R3	256	19	-0.2
	R6	64	19	0.2
	R7	256	19	0.1
	R8	128	19	0.2
	R9	64	19	-0.2
	R10	128	19	0.1
	R11-R17	256	19	0.7

* The RF titres were equal for free and hidden RF (see Methods); that is, none of the sera contained demonstrable hidden IgM RF.

There was no relation between the regression coefficients and RF titres (Table III); that is, the amount of precipitates obtained for 0.4 and 0.6 mg IgM did not vary with the serum level of the RF titre.

The next step was quantitative precipitation of four seronegative RA sera (Table IV). Two of the sera showed RF activity with a method that detects hidden RF. In the other two sera no RF activity could be detected by this method. Hidden RF was, in these cases, demonstrated

TABLE IV. Quantitative precipitation of RA sera containing hidden RF IgM with rabbit IgG anti-Fc μ : regression coefficients (K ; see Table II) obtained for RF IgM with different titres of hidden RF IgM (see Methods)

Sample	Titre		K
	Free RF	Hidden RF	
R18	0	0*	-0.3
R19	0	0*	0.2
R20	0	1024	0.1
R21	0	256	0.2

* Hidden RF was demonstrated after the serum had been treated with dissociating agents and gel filtered (see Methods).

with the test that detects such RF only after the serum had been treated with dissociating agents and gel filtered. Positive regression coefficients were obtained in three of the cases with hidden RF. One sample gave a slightly negative K value. Thus, positive or slightly negative regression coefficients were found for those sera that contained hidden RF (R20 and R21) or strongly hidden RF (R18 and R19).

The results of the quantitative precipitin reaction of all the RA sera are compared with those of normal sera in Table V. The mean regression coefficients were negative for the normal sera but positive for the RA sera. In those cases where the regression coefficients were negative for the RA sera, they were always higher than the corresponding coefficients for normal sera (cf. Tables III and IV).

Seven IgG anti-Fc μ were used in this study. The anti-Fc μ were used separately after they had been adjusted to the same antibody titre. To check how the use of the different anti-Fc μ affected the precipitin reaction, the precipitin curves for one and the same serum with different anti-Fc μ were compared. The amount of pre-

cipitates varied with the anti-Fc μ used. This variation was observed in all three precipitin zones. Roughly the same difference in amount of precipitates between two anti-Fc μ was observed in the zones of a slight and a higher antigen excess. The regression coefficients calculated from the amount of precipitates at 0.4 and 0.6 mg IgM were therefore similar for a given serum, when analysed with the various anti-Fc μ ; that is, a positive K value did not change to a negative one, and vice versa, when different anti-Fc μ were used.

A model was constructed from all the precipitin reaction data with IgG anti-Fc μ obtained for all RF IgM and normal IgM (Fig. 4). The amount of precipitates corresponding to 0.08 mg IgM and 0.4 mg IgM was calculated as the mean values for all analyses of RF IgM and normal IgM with the various anti-Fc μ . The points a and o thus represented all RF IgM and normal IgM that had been analysed. The point b (0.6 mg IgM) was obtained in the same way for all RF IgM and the point c (0.6 mg IgM) in the corresponding manner for all normal IgM. The regression coefficient for RF IgM (K_{RF}) varied between -0.25 and 2.05 and for the normal IgM (K_N) between -3.85 and -0.40.

Our material was thus clearly organized in such a manner that RF IgM and normal IgM produced significantly different K values as a result of differences in precipitin behaviour with IgG anti-Fc μ in the zone of higher antigen excess.

DISCUSSION

The precipitin curves obtained with IgG anti-Fc μ were the same up to the zone of slight antigen excess for the normal and the RA serum pool. The RA sera contained, besides RF IgM, normal IgM that could react with anti-Fc μ . The amounts of precipitates in the zones of antibody and antigen excess have been shown to increase when RF IgM is added to immune complexes, probably because RF IgM binds to the complexes [31]. The similarities in the amounts of precipitates up to slight antigen excess, as observed in this study, thus indicated that RF IgM did not react with complexes of normal IgM and IgG anti-Fc μ owing to its anti-IgG activity but reacted with anti-Fc μ in the same way as normal IgM in the zones of antibody excess and slight antigen excess.

TABLE V. Quantitative precipitation of IgM in RA sera and normal sera with rabbit IgG anti-Fc μ : comparison of regression coefficients (K ; see Table II)

Sample	K
All RA sera; mean value	0.3
N1	-3.9
N2	-1.4
N2-N8	-1.2
N9-N12	-0.4

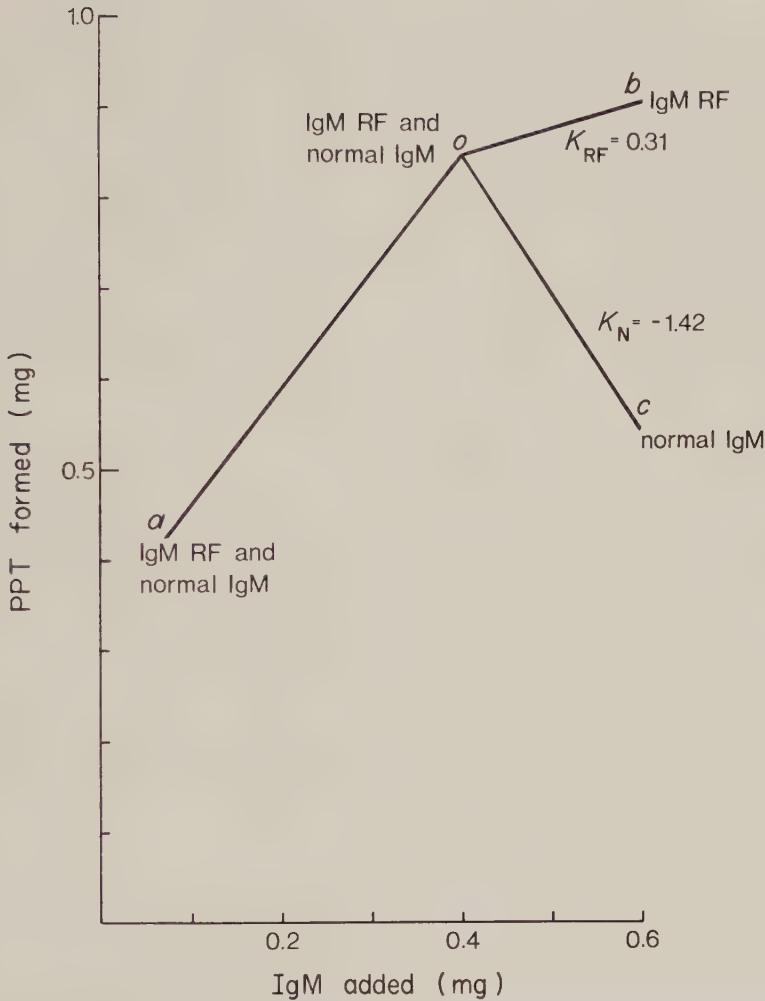


FIG. 4. A model for a quantitative precipitin curve constructed from the analyses performed in this investigation. The mean regression coefficient for the RA sera (K_{RF}) and for the normal sera (K_N) is given.

However, in the zone representing a higher excess of antigen more precipitates formed for IgM in the RA sera than for IgM in the normal sera. RF IgM thus seemed to react with immune complexes in this zone. It therefore seemed plausible that RF IgM, in this part of the antigen excess zone, reacted with the $Fc\gamma$ part of the antibodies in the immune complexes already formed. This conclusion was strengthened by the fact that different amounts of precipitates were obtained in the precipitin analysis of normal and RA serum when intact IgG anti- $Fc\mu$ were used but similar amounts of precipitates were formed when $F(ab')_2\gamma$ anti- $Fc\mu$ were used, and by the fact that RF IgM did not have a

greater ability to precipitate rabbit IgG than did normal IgM.

The IgM contents in the supernatants from the precipitin reactions with IgG anti- $Fc\mu$ were the same for RA and non-RA serum. The large difference in amount of precipitates obtained for these sera could therefore not be explained by incorporation of different amounts of IgM into the precipitates. This indicated that precipitates were formed owing to a rearrangement in the complexes. Such a rearrangement could occur, for example, if normal IgM dissociated from the complexes of IgM and IgG anti- $Fc\mu$ when the RF IgM was added in the zone of antigen excess. This would be in accordance with the

results obtained earlier and showing that the amount of free antigen increases in the antigen excess zone when RF IgM is added to immune complexes [2].

The increased precipitation observed for RF IgM in the antigen excess zone indicated that the complexes formed when the Fab μ part of RF IgM reacted with the Fc γ part of the IgG anti-Fc μ in the immune complexes were less soluble than the complexes containing only the Fc μ -Fab γ bonds (normal IgM). This was in accordance with results obtained for equine IgG and IgG (T), showing that reactions that favour a binding between a single antibody and a single antigen form less precipitates with that antigen than do reactions that favour cross-linking between several antigens and antibodies [23].

Strongly hidden RF IgM and free RF IgM with demonstrable anti-IgG activity gave similar precipitin curves. This could be explained by the assumption that all RF IgM had the same conformation in the Fc μ part. It could also be assumed that free RF IgM formed Fab μ -Fc γ bonds during the precipitation, whereas such bonds were originally contained in the hidden RF IgM. The presence of such bonds could result in a lower solubility in water, i.e. the formation of more hydrophobic molecules. Our conclusion was that it was not a demonstrable RF activity but rather the hydrophobicity or the size of the molecules that was necessary to obtain more precipitates in the antigen excess zone. This conclusion was verified by the results obtained on quantitative precipitin analysis of isolated macroglobulinaemic IgM without RF activity, as two rapidly sedimenting IgM gave the same deviations in the antigen excess zone as RF IgM, whereas four isolated 19S IgM gave results similar to those obtained with normal human serum.

The amount of precipitates obtained with a given amount of IgM varied with the IgG anti-Fc μ used. Such differences in precipitin reaction have been demonstrated, for example, when different rabbit anti-fibrinogens precipitated fibrinogen [11] and when rabbit antibodies against human Fab γ , C γ 2 or C γ 3, respectively, precipitated intact human IgG [3]. In this study the regression coefficients for the lines representing the precipitation in the antigen excess zone obtained for different IgG anti-Fc μ with the same antigen were largely the same. This was also the case for some of the antisera studied by

Fritz *et al.* [11]. This meant that even when seven different anti-Fc μ were used, differences in the above-mentioned regression coefficients could still be used to distinguish RF IgM and normal IgM.

One of the normal serum pools had a regression coefficient that was not significantly different from those obtained for three of the RA sera. This could be explained by the demonstrated occurrence of antiglobulins also in normal sera [18], and our conclusion was that a 'high' regression coefficient for normal serum indicated the presence of RF IgM in such serum.

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Isolation and Characterization of Intermediate Complexes and Other Components with Common Antigenic Determinants

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Fractions with affinity for heat-denatured human IgG (HDG) were isolated from four sera that contained intermediate complexes (IC). These IC fractions contained part of the 7S IgG, all IC, and part of the rapidly sedimenting complexes (RC) found in the sera. The IC consisted of IgG1 or IgG3 and the RC of IgG and IgM with kappa and lambda light chains. The IgG in the IC fractions contained an abnormally large amount of neuraminic acid. No correlation between IgG subclass or content of neuraminic acid and complex formation was found. There were indications that the formation of IC was not only the result of a self-association of IgG molecules with anti- γ -globulin activity. Specific rabbit antisera were prepared against two of the IC fractions. Affinity chromatography with immobilized IgG and F(ab')₂ γ from these antisera confirmed the presence of common antigenic determinants in the sera. These determinants occurred mainly in 7S components in one individual, in IC in one and in RC in another. Only a minor part of the serum components with affinity for HDG contained the determinants. RF activity was found in the components that lacked and in those that contained the common antigenic determinants.

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Intermediate complexes (IC) have been demonstrated in sera of patients with rheumatoid arthritis (RA) and of those with hypergamma-globulinaemic purpura [24, 35, 40].

The IC in three RA sera were formed by self-association of IgG rheumatoid factors (IgG RF) [36], and the IC in one non-RA serum were formed due to IgG aggregation rather than to an antigen-antibody reaction [16].

IgG in IC differs from normal IgG. A restriction to subclass 1 [15] and an abnormally large content of neuraminic acid [17] have been reported. Differences between IgG RF and normal IgG have also been demonstrated. IgG1 is more dominant in IgG RF than would be expected from the subclass distribution in normal human IgG [2]. The fragments obtained on papain hydrolysis of IgG RF and normal IgG are different [46], and so are the circular di-

chromism spectra of IgG from RA and non-RA patients [21].

Unique antigenic determinants present in a group of molecules with similar function have been referred to as cross-idiotypic [22]. Such determinants occur in human monoclonal IgM RF [23] and are shared by monoclonal and polyclonal IgM RF and by monoclonal IgG RF and polyclonal IgM RF [5]. Attempts have been made to prepare antibodies to human polyclonal IgG RF [20] and human IgG dimers from RA serum [36], but to our knowledge no reports are available about common antigenic determinants in polyclonal IgG RF or IgG complexes with RF activity.

In this study immunoglobulin complexes were isolated from four sera that contained intermediate complexes. The isolated fractions were characterized with regard to RF activity,

dissociation and association of the complexes, IgG subclass distribution, and neuraminic acid content. Rabbit antibodies were then prepared which reacted specifically with unique antigenic determinants in some 7S components, some intermediate complexes, and some rapidly sedimenting complexes.

MATERIALS AND METHODS

Sera. Sera from four patients with rheumatoid arthritis (RA sera; OS and ED) or hypergammaglobulinaemic purpura (non-RA sera; MG and BL) were analysed. Three of the patients (OS, ED and BL) also had a hyperviscosity syndrome, and one (ED) also had hypergammaglobulinaemic purpura. Normal sera from registered blood donors were used as controls. The sera had been stored at -20°C .

Antisera against normal human serum proteins. Anti-kappa, anti-lambda, anti-Fab γ , anti-Fc γ , and anti-Clq were from Behringwerke AG, Marburg, West Germany. Anti-whole human serum was from DAKO-immunoglobulins Ltd, Copenhagen, Denmark, or from Hyland, Division Travenol Labs. Inc., Los Angeles, Calif., USA. Anti- α -lipoproteins and anti- β -lipoproteins were from the Department of Clinical Chemistry, University of Lund, Lund, Sweden. Anti-IgG1, 2, 3 and 4 were from Nordic Immunol. Labs., Tillburg, The Netherlands. Anti-IgG, anti-IgM and anti-IgA were prepared in our laboratory by hyperimmunizing with the purified immunogen in complete Freund's adjuvant and were appropriately absorbed. All antisera except the IgG sub-class-specific ones (swine) originated from rabbits.

Isolation of human and rabbit IgG. Human IgG was a commercial preparation for human use (AB Kabi, Stockholm, Sweden). 7S IgG was obtained from this preparation by gel filtration on Sephadex G-200 in 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0. Human myeloma 7S IgG of subclass 1, 2 and 3 was isolated as described earlier [12].

Rabbit IgG was prepared from serum by salting out with 1.84 M $(\text{NH}_4)_2\text{SO}_4$ overnight at 4°C . The washed precipitates were dissolved and dialysed against 0.16 M NaCl. The non-specific rabbit IgG was then applied to a DEAE-cellulose column, from which the IgG was eluted with 0.02 M sodium phosphate, pH 8.0.

Isolation of IC fractions. Sepharose 6B containing 500 mg immobilized [4] heat-denatured (63°C , 30 min) human IgG (HDG) was used. Sequential elution was made with 0.075 M Tris, 0.075 M NaCl, 10^{-3} M CaCl_2 , pH 8.1; 1 M NaCl; and 0.2 M 2-aminobutane, 0.075 M Tris, 10^{-3} M CaCl_2 , pH 11.0. The eluates were concentrated by vacuum dialysis in collodion bags against 0.16 M NaCl, 0.1 M glycine, pH 7.0, to avoid non-specific aggregation and precipitation [11]. IC fraction referred in all cases except BL (pool of delayed Tris fractions) to the 2-aminobutane pool. The maximum binding capacity of the columns was never exceeded.

Preparation of specific antiserum against antigenic determinants in the IC fraction. Fractions from BL and ED were used to hyperimmunize ten rabbits

each. 1 mg immunogen dissolved in 0.5 ml 0.16 M NaCl, 0.1 M glycine, pH 7.0, was mixed with 0.5 ml complete Freund's adjuvant (Difco, Detroit, Mich., USA) and was injected at several sites behind the scapulae of each rabbit. The rabbits were given booster doses in the same way when required. A part of each antiserum pool was absorbed with normal human serum. After this absorption four of the antisera contained antibodies that precipitated with the immunogen. Two were used for preliminary experiments only, and the other two, which had higher titres, were used in the study. The antiserum a-BL was absorbed on a larger scale by dropwise addition of normal human serum. The final absorption of a-BL and the sole absorption of a-ED were performed with affinity chromatography to immobilized normal human serum coupled [4] to Sepharose CL-4B. IgG was isolated from the antisera before the antibodies were applied to the immunosorbent columns. The antibody fraction that lacked affinity to the ligand was eluted with 0.075 M Tris, 0.075 M NaCl, 10^{-3} M CaCl_2 , pH 8.1, and concentrated by vacuum dialysis as described under IC fractions. The specificity of the absorbed antibodies was assessed by double immunodiffusion against normal human IgG, normal human serum, and the immunogens over a wide range of concentrations (0.02–400 g protein/l). Precipitates were obtained only with the immunogens, and BL could be diluted to at least 0.02 g/l and ED to at least 0.08 g/l without disappearance of the precipitation with undiluted a-BL and a-ED, respectively.

Enzymatic digestions. Pepsin digestion was performed as described by Turner *et al.* [44] and papain digestion according to Porter [37]. The digestions were checked by ultracentrifugal analysis and by immunoelectrophoresis against anti-Fab γ and anti-Fc γ . After papain digestion more than 95% of the material in OS, BL and MG had a mean sedimentation coefficient (s_{20w}) of 3.35S–3.85S (± 0.10) (depending on the protein concentration). The IC fraction ED contained 25% IgM, and a corresponding amount of 5S components was found on ultracentrifugation of the papain-digested ED. The amounts of Fab γ and Fc γ were estimated by measuring the density of the agarose gel electrophoretic bands by a densitometer.

Neuraminidase treatment was performed in 0.05 M NaAc, 0.05 M CaCl_2 , pH 5.5. Four units of neuraminidase was added per milligram protein, and the sample was incubated at 37°C overnight. The enzyme reaction was inhibited by dialysis against 0.05 M Tris, 0.01 M EDTA, pH 8.2 [1]. The effectiveness of the reaction was checked with agarose gel electrophoresis.

Isolation of $\text{F(ab')}_2\gamma$ fragments. Commercial normal human IgG, IgG from a-BL, a-ED and IgG from non-immunized rabbits were pepsin-digested, applied to a column of Sephadex G-200, and eluted with 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0. The appropriate components were selected. The absence of undigested 7S IgG was checked by analytical ultracentrifugation against a standard human 7S IgG preparation and by immunoelectrophoresis. In these experiments the protein concentration in the $\text{F(ab')}_2\gamma$ fractions was 10 g/l, and control experiments showed that 2% IgG in the samples would be detected.

Isolation of serum proteins with affinity to immobilized IgG or $\text{F(ab')}_2\gamma$. The IgG or the $\text{F(ab')}_2\gamma$ was coupled

[4] to Sepharose 6B. Elution and concentration of the fractions was done as described under isolation of IC fractions.

Ultracentrifugal analysis. Ultracentrifugal analysis was done with an An-D rotor at 59,780 (259,700 g) rpm in a Beckman Analytical Ultracentrifuge Model E with Schlieren optics. The temperature was 20°C, and the sample was dissolved in 0.16 M NaCl, 0.1 M glycine. The total protein concentration was always below 20 g/l, and no correction was made for the Johnston-Ogston effect. s_{20W} values were obtained by making the usual correction for density and viscosity using a $\bar{v}$ value of 0.723 [31]. $s_{0,20W}$ values were determined as described previously [12].

The s-rate distribution of the components in the samples was determined as described previously [10].

In experiments determining the effect of additional IgG on the distribution of components in the IC fractions an amount of IgG equal to that originally present in the IC fraction was added.

Qualitative and quantitative protein determinations. The protein concentration in all eluates and fractions was assayed by the light absorbancy at 280 nm ($E_{1\%}^{1cm}$ at 280 nm of 12.5) [29]. The serum protein concentrations were determined according to Lowry *et al.* [27]. Electroimmunoassay was used to quantify Clq and single immunodiffusion to quantify IgG and IgM. Owing to variation of the diffusion rate between 7S IgG and IgG-IgG complexes, the errors in the determination of the IgG concentration was $\pm 5\%$. IgM was quantified in the reduced samples (0.1M β -mercaptoethanol, 4°C, overnight) with an error of less than $\pm 3\%$. The errors were expressed as maximum deviation from the mean.

Single immunodiffusion was performed according to Mancini *et al.* [28], double immunodiffusion according to Ouchterlony [34], immunoelectrophoresis according to Scheidegger [41] and electroimmunoassay according to Laurell [26]. These analyses and the agarose gel electrophoresis were run in 0.8% agarose gel in 0.075 M barbital buffer, 2 mM calcium lactate, pH 8.6. The calcium lactate was excluded in the Clq analyses.

RF activity. The RF activity of sera and IC fractions was determined with the acryl fixation test according to Winblad [48]. The RF activity of fractions isolated from the immobilized F(ab')₂γ fragments of a-BL and a-ED was determined with a modified Waaler-Rose test [13] in which EDTA, glycine and NaCl were used to liberate hidden rheumatoid factors and to inactivate the complement. The RF activity in the a-BL and a-ED antibodies was determined with a latex fixation test, modified in the corresponding manner [13]. The reaction was always considered positive when the titre was at least 60.

Major IgG subclass. The major IgG subclass was established by high-voltage electrophoresis [7] and immunoelectrophoresis on papain-digested samples [39]. Double immunodiffusion and immunoelectrophoresis against IgG subclass-specific antisera were used to check whether the specificity of these antisera corresponded to the results obtained on estimation of the major subclass and to ascertain the presence or absence of minor subclasses.

Neuraminic acid content. The neuraminic acid content was determined according to Warren [45]. The error

was $\pm 7\%$. In the IC fraction ED 25% (w/w) of the proteins were IgM. The neuraminic acid content per mole of 7S IgG (x) was in this case calculated according to the formula $0.75 \times x + 0.25 \times 6 = \text{moles of neuraminic acid per mole 7S component}$ (assuming six residues of neuraminic acid per 7S unit of IgM [30]).

RESULTS

The distribution of intermediate (IC) and rapidly sedimenting (RC) complexes in the four sera is given in Table I. The two RA sera, OS

TABLE I. Distribution of intermediate and rapidly sedimenting complexes in the sera (% of total protein)

Serum	Intermediate complexes		Rapidly sedimenting complexes	
	9S	> 9S < 19S	19S	> 19S
OS*	7	20	0	2
ED*	15	3	0	27
MG	0	6	2	0
BL	8	0	3	0

* Sera from the patients with rheumatoid arthritis and a positive rheumatoid factor titre.

and ED, contained IC with two s-rates and RC, whereas the two non-RA sera contained IC with only one s-rate.

To isolate the IC and the RC, the sera were applied to columns containing immobilized heat-denatured human IgG (HDG). Fractions without affinity were invariably eluted as a large single peak in Tris buffer, pH 8.1. Besides this major peak BL showed a second peak. 40% of the proteins in serum BL were found in this delayed Tris buffer fraction. No proteins were in any case eluted with 1 M NaCl. 30% of the proteins in OS, 26% of the proteins in ED, 22% of those in serum MG, and 5% of those in normal serum had a high affinity for the label and were eluted with the third elution buffer, the 2-aminobutane buffer, pH 11.0. The proteins in the fractions with affinity—that is, the 2-aminobutane fractions, and, in the case of BL, the delayed Tris buffer fractions—migrated in the γ -region on agarose gel electrophoresis. They were RF-positive (titre ≥ 60), although

TABLE II. Distribution of components in the fractions with affinity to heat-denatured human IgG (HDG) (% of total protein)

Sample	Obtained distribution*				Predicted distribution†			
	7S	9S	> 9S ≤ 19S	> 19S	7S	9S	> 9S ≤ 19S	> 19S
OS	3	5	83	9	4	25	65	6
ED	5	42	16	37	0	59	12	29
MG	82	0	16	2	68	0	32	0
BL	60	40	0	0	73	20	7	0

* Obtained by ultracentrifugal analysis of the fractions with affinity to HDG, i.e. the IC fractions.
† Obtained by ultracentrifugal analyses of serum and the corresponding fractions without affinity to HDG. The predicted distribution was obtained by subtracting the amount of the different components in the fractions without affinity to HDG from the amount of the corresponding components in the serum.

only the RA sera OS and ED had shown RF activity.

Ultracentrifugal analyses showed that IC existed only in the fractions with affinity for HDG. The s-rate distribution of components in the IC fractions is given in Table II. Besides IC and RC, the fractions from the RA sera contained traces and those from the non-RA sera large amounts of 7S components.

The changes, if any, in 'composition' of the complexes due to isolation were determined from the s-rate distribution in serum and the isolated fractions by comparing the obtained and the predicted distributions (Table II). On isolation some of the complexes aggregated in OS and ED but dissociated in MG and BL.

All the IC fractions contained both kappa and lambda light chains. Not only IgG and IgM but also α- and β-lipoproteins (1%) and Clq were found. The IC in three of the fractions consisted mainly of IgG; that is, more than 90% of the proteins were IgG and less than 4% was IgM. The fourth fraction, ED, contained 25% IgM besides the IgG, so that no definite conclusion about the composition of the IC could be drawn. In all the cases except BL, the major IgG was of subclass 1, with traces of IgG2 and IgG3. BL contained IgG3 and traces

of IgG1 and 2. The Clq content in the sera and IC fractions is given in Table III. The IC fractions OS and MG contained almost all, and the fractions ED and BL only part of, the Clq found in serum.

The effect of additional 7S components of normal human IgG or myeloma IgG1, 2 and 3 on the complex formation was also assessed. Ultracentrifugal analysis of the IC fractions showed that after the additions the amount of IC increased by more than 25% and the amount of 7S components decreased by more than 10%. In the non-RA samples the opposite occurred. The amount of IC decreased by more than 25% at the same time as the amount of 7S components increased by more than 10%. There was no correlation between the observed effect and the subgroup of the added IgG.

Determinations were made also of the affinity for immobilized normal human 7S IgG and for F(ab')₂γ fragments thereof. No proteins were ever found in the NaCl fractions. When serum BL was applied to the F(ab')₂γ column, no proteins were detected as a delayed Tris fraction or in the 2-aminobutane buffer. But when intact IgG was used, 10% of the applied proteins were found in the 2-aminobutane buffer and 47% as a delayed Tris fraction. Of the proteins in serum ED 2% had affinity for F(ab')₂γ and 24% for the intact IgG.

The neuraminic acid content was abnormally high in all the IC fractions. Normal human IgG contained 0.7, OS 3.2, ED 2.7, MG 2.4, and BL 2.7 mole of neuraminic acid per mole IgG.

Ultracentrifugal analysis showed that neither the complex formation nor the reactivity with

TABLE III. Clq content in sera and intermediate complex (IC) fractions (g/l)

	OS	ED	MG	BL
Serum	0.38	0.40	0.17	0.77
IC fraction	0.32	0.10	0.18	0.11

HDG was affected when the fractions were treated with neuraminidase.

The IC fractions were papain-digested. They were re-applied to the immobilized HDG after the effectiveness of the hydrolysis had been checked. The results are given in Table IV.

TABLE IV. Amount of Fc γ and Fab γ fragments that had affinity for heat-denatured human IgG when the intermediate complex fractions had been papain-digested (% of total amount of the same fragment)

	OS	ED	MG	BL
Fab γ	70	50	75	25
Fc γ	25	80	25	95

Since no proteins were eluted with NaCl or as a delayed Tris buffer fraction, proteins with affinity were eluted with 2-aminobutane buffer and fractions without affinity as major Tris fractions. A large part of the Fab γ fragments in the IC fractions had no affinity for HDG. Various amounts of the Fc γ fragments were found in fractions without affinity.

Antisera were thereafter prepared against the IC fractions BL and ED. The absorbed antisera (a-BL and a-ED) precipitated proteins in the immunogenic fraction but not in other fractions isolated from the corresponding serum; that is, the precipitin reaction was restricted to the proteins with affinity for immobilized HDG. Native or heat-denatured normal human IgG did not precipitate a-BL and did not inhibit the precipitation between the immunogenic BL fraction and a-BL. The antisera showed no RF activity. The IC fractions did not precipitate rabbit γ -globulins isolated from non-immunized rabbits.

IgG from a-BL and a-ED was isolated and immobilized. 1% of the proteins in normal human serum had affinity for these labels (Table V). More than 10% of the proteins in all the sera, except ED, had affinity for both labels. More proteins in ED had affinity for the a-BL than for the a-ED IgG. Immunodiffusion showed that the proteins that had affinity for immobilized a-BL were mainly IgG, whereas those with affinity for a-ED were IgG and IgM.

F(ab') $_2\gamma$ fragments prepared from an IgG isolated from non-immunized rabbits were thereafter immobilized, and the affinity to this

TABLE V. Amount of serum proteins with affinity for immobilized IgG of a-BL and a-ED (% of total serum protein)

Serum	a-BL IgG	a-ED IgG
NS	1	1
OS	12	13
ED	18	4
MG	12	12
BL	40	27

label was determined. Proteins in normal human serum or serum BL had no affinity, whereas 1% of the proteins in serum OS and 2% of the proteins in serum ED had affinity.

F(ab') $_2\gamma$ fragments prepared from the a-BL and a-ED IgG were then immobilized. The results obtained when the affinity for these labels was determined are given in Table VI.

TABLE VI. Amount of serum proteins with affinity for immobilized F(ab') $_2\gamma$ of a-BL and a-ED (% of total serum proteins)

Serum	a-BL F(ab') $_2\gamma$	a-ED F(ab') $_2\gamma$
NS	0.3	0.4
OS	3.3	4.0
ED	10.1	6.5
BL	9.8	1.3

Serum MG could not be analysed because of scarcity of material. Except for the ED/a-ED pair, the amount of proteins with affinity for F(ab') $_2\gamma$ was lower than the amount that had affinity for the intact antibodies (compare with Table V). For serum ED the amount with affinity for the a-ED F(ab') $_2\gamma$ was larger than that with affinity for the a-ED IgG.

The s-rate distribution of components in the fractions that had affinity for the F(ab') $_2\gamma$ fragments of a-BL and a-ED is given in Table VII. For one and the same serum the proteins that had affinity for the a-BL and a-ED fragments, respectively, had a remarkably similar s-rate distribution. Both the OS fractions thus contained mainly IC, both the ED fractions mainly RC, and both the BL fractions mainly 7S components. The fractions from normal serum contained mainly 7S components. Ultracentrifugal analysis of the fractions without affinity for the F(ab') $_2\gamma$ fragments confirmed

TABLE VII. S-rate distribution of the serum components with affinity for immobilized $F(ab')_2\gamma$ of a-BL and a-ED (% of total protein)

Sample with affinity for:		7S	9S < 19S	> 19S
a-BL $F(ab')_2\gamma$	a-ED $F(ab')_2\gamma$			
OS		42	58	0
	OS	31	69	0
ED		0	0	100
	ED	0	0	100
BL		100	0	0
	BL	100	0	0

the data showing that only a part of the 7S, IC, and RC components in the sera had affinity for the column ligand.

There was no relation between RF activity and affinity for the immobilized a-BL and a-ED $F(ab')_2\gamma$ fragments. RF activity was found both in fractions with and in fractions without affinity for these fragments. None of the BL fractions had any RF activity, which should be compared with the low RF activity found in BL fractions with affinity for HDG.

Agarose gel electrophoresis showed that the proteins in the fractions with affinity for the $F(ab')_2\gamma$ fragments of a-ED and a-BL migrated in the γ -zone. The precipitin arcs obtained on immunoelectrophoresis against anti-whole human serum were without exception found in the same region. Single and double immunodiffusion showed that the fractions from BL contained mainly IgG and small amounts of IgM (1%). The fractions from OS and ED contained mainly IgG, but also IgM (12–31%). All fractions contained both kappa and lambda light chains. All fractions, except BL from a-ED $F(ab')_2\gamma$, contained Clq, as did all the fractions without affinity.

To determine what part of the IgG molecule had affinity for a-BL, the components in serum BL with affinity for the a-BL $F(ab')_2\gamma$ were pepsin-digested, and the $F(ab')_2\gamma$ components were isolated and run again on the column. All $F(ab')_2\gamma$ had affinity; that is, the antigenic determinants were located in the $F(ab')_2\gamma$ fragments of the molecules.

DISCUSSION

The nature of the solvents may affect the poly-

merization of anti- γ -globulins [36]. To facilitate a comparison of the isolated fractions, the same solvents and immunosorbent technique were used to isolate IC and other serum components from all the sera.

We found no evidence that the affinity for the immobilized proteins depended on non-specific interactions. The small amount of components in normal human serum with affinity for, for example, HDG was compatible with the demonstrated occurrence of anti-globulins also in normal human sera [14]. Our results also confirmed those obtained by Nardella & Mannik [32] showing that the non-immunospecific interactions of IgG and IgG-agarose are weak or non-existent at pHs above 4 and ionic strengths corresponding to an NaCl molarity of 0.5.

The IC fractions isolated from the non-RA sera contained a large amount, and those from the RA sera a small amount, of 7S components with affinity for HDG. The RA sample ED contained more than 5% of the proteins as IgM, and 2% of the proteins in this sample had affinity for $F(ab')_2\gamma$ of normal IgG. Self-association of IgG molecules with anti- γ -globulin activity might be inhibited by IgM (> 5%) and should not be expected when the anti- γ -globulin molecules show anti-Fab γ activity [36]. The existence of 7S IgG in fraction ED could thus be explained by an anti-Fab γ activity or a high content of IgM. The two non-RA samples, which contained large amounts of non-aggregated IgG with affinity for HDG, contained less than 4% IgM and no anti- γ -globulins with anti-Fab γ activity. This indicated mechanisms other than self-association for the complex formation.

All of the monomeric IgG and the IC in

serum BL had affinity for HDG when columns containing HDG bound to Sepharose were used. No agglutination was observed, however, when serum was added to latex particles coated with an identical HDG. One explanation of these differences could be that the binding sites of the Fab γ with affinity for HDG were occupied in the serum but became free on the gel filtration in the chromatography column. This could also explain why some Fab γ fragments obtained after papain digestion had a higher affinity for immobilized HDG than had the intact IgG molecules and IC.

The IC in all the sera had affinity for HDG. The isolated complexes were, however, affected in different ways when native human IgG was added. The IC from the non-RA sera dissociated and the IC from the RA sera combined with the added IgG. Differences in reactivity thus occurred among groups of sera related to different diagnoses, as was also suggested by Halla *et al.* [9]. Differences in affinity between Fab γ and Fc γ within the complexes isolated from sera from individuals with the same diagnosis might, however, also occur. The interactions between the Fab γ and Fc γ in our IC fractions from the RA patients were weak or non-existent, but they were relatively high in the IC fractions from the RA patients studied by Pope *et al.* [36].

After the IC fractions had been papain-digested, Fab γ fragments without affinity for HDG were found in all the fractions. No intact IgG molecules were detected in the fractions. IgG has two identical Fab parts [43]. The complexes with affinity for HDG therefore contained more than one population of IgG molecules; one population contained Fab γ with affinity for HDG and the other contained Fab γ without such affinity.

Clq has affinity for immune complexes greater than 20S, for aggregated human IgG, for rheumatoid factors, and for aggregates composed of RF and soluble complexes [18, 42, 49, 50]. Most of the Clq was, as expected, recovered in those fractions that had affinity for HDG. However, part of the Clq in the ED serum had no affinity for HDG or those complexes that reacted with HDG. Clq could thus in this case be divided into subpopulations with different affinities. One part of this Clq was thus similar to that described by Chapuis *et al.* [3]. The Clq studied by them origin-

ated from an individual with severe lupus-like syndrome, and it also lacked ability to bind to Sepharose-IgG. Some Fc γ fragments from the papain-digested IC fractions were found in fractions with affinity for HDG. One plausible explanation of this was that Clq that had bound to HDG also combined with Fc γ in the applied fractions because Clq has several binding sites for Fc γ [38]. Any specific interaction between Fab γ and Fc γ was excluded because the monovalent Fab γ that bound HDG could not at the same time bind the Fc γ fragments.

As reported by others [2, 15], we also found that IgG1 dominated in three of our IC fractions. The effect of additional IgG on the dissociation/association of the complexes was not related to the subclass of the added IgG. The implication of the subgroup restriction and its relation to the complex formation therefore remained unclear.

The IgG in the IC fractions contained more neuraminic acid per mole than normal IgG. The same result has been obtained previously for the IC from a patient with RA [17]. The reassociation of these IC required the presence of neuraminic acid [17]. This was not the case for the IC studied here. Our proteins did not contain cryo-IgG, and they were not myeloma IgG—proteins that have been shown to contain an abnormal content of neuraminic acid [30, 51]. The significance of the high content of neuraminic acid in our samples thus remained unclear.

Complete Freund's adjuvant was used for the immunizations of rabbits with the IC fractions. The adjuvant effect is mediated by a peptidoglycolipid of *Mycobacterium tuberculosis* [47]. The injection of lipopolysaccharides might, in mice, elicit the production of rheumatoid factors that cross-react with human IgG [19]. The antisera obtained here did not react with latex particles coated with HDG or with normal IgG. We therefore had no reason to assume that our antisera reacted with the immunogenic IC fractions because of an anti-IgG activity of the antibodies.

The amount of proteins with affinity for the intact antibodies was much larger than that with affinity for the F(ab') $_2\gamma$ of the same antibodies. Intact antibodies could therefore not be used to isolate components containing the common antigenic determinants. This was so even when the antisera had been absorbed on

immunosorbent columns and thus did not contain immune complexes due to overabsorption.

A small amount of proteins in all the sera had affinity for $F(ab')_2\gamma$ from non-immunized rabbits. Since this reactivity could partly be dependent on secondary reaction between complexes bound to $F(ab')_2\gamma$ and RF active complexes in solution, the absolute amount of proteins with $F(ab')_2\gamma$ affinity could not be calculated. But, because the amount that reacted with the non-specific $F(ab')_2\gamma$ was so small compared with that with affinity for the $F(ab')_2\gamma$ from the specific antibodies, the possibility that the reaction with the $F(ab')_2\gamma$ of the specific antisera depended on 'non-specific interactions' could be eliminated.

Clq was detected in all the fractions that lacked affinity for the immobilized $F(ab')_2\gamma$ from the specific antibodies, but not in all the fractions that had affinity for this label. This showed that Clq was not the antigen and that the combination with the specific antibodies did not occur through Clq.

Not only components in the sera from which the immunogenic molecules originated but also components in the other IC sera had affinity for the $F(ab')_2\gamma$ from a-ED and a-BL. It was interesting to note that the components with this affinity in serum ED were mainly larger than 19S (RC), in serum OS mainly of intermediate size (IC), and in serum BL mainly of the 7S type. This was true whether $F(ab')_2\gamma$ from a-ED or from a-BL was used as immunosorbent. Only a part of the RC, IC or 7S components found in the sera had the above-mentioned affinities. The actual antigen(s) was thus only exposed in some of the RC, IC or 7S molecules. The occurrence of this antigen was related to components of a special 'size' in one person but not to the 'size' of the components in another person.

The presence of immunogenic determinants in the $F(ab')_2\gamma$ part has been used as a criterion of idiotypic specificity [8], and such determinants also often reflect the specificity of antibodies [33]. Cross-idiotypic determinants have recently been described on polyclonal IgM RF and monoclonal IgG RF [5]. In the latter case $F(ab')_2\gamma$ fragments of the anti-idiotypic antibodies blocked the Waaler-Rose reaction of the RF active molecules, which was taken as an indication that the antisera were directed against determinants in the antigen-binding

site of the RF [5]. It was shown that the $F(ab')_2\gamma$ fragments from BL were those that had affinity for the specific antibodies, but we found no relation between RF activity and reactivity with the specific antisera. Both fractions with and fractions without affinity for the $F(ab')_2\gamma$ of the specific antisera showed RF activity. We could therefore not exclude the possibility that the reaction was not with RF-positive proteins but with molecules binding such proteins.

Both V_K and V_H subgroup restrictions have been demonstrated for IgM RF [6, 25]. Førre *et al.* have demonstrated shared idiotypic antigens between certain IgG and IgM RF, but no V_H subgroup restriction among these proteins [5]. Our fractions that contained the common antigenic determinants contained IgG and IgM. The relationship between these antigenic determinants and those found in RF by Førre *et al.* [5] remains to be investigated. No information is given about whether their anti-idiotypic antisera reacted with all molecules with RF activity.

The IC sera contained unique immunoglobulins with common antigenic determinants. These common antigenic determinants occurred both in 7S components and in IC and RC and could in one case be demonstrated in the Fab γ part of the 7S molecules with affinity for the specific antisera. Work is under way to relate the common antigenic determinants to a certain part of the reactive IC and RC molecules.

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CIRCULAR DICHROISM OF IMMUNE COMPLEXES WITH RHEUMATOID FACTOR ACTIVITY

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Abstract—The circular dichroism (CD) of IgG complexes with RF activity and that of monomeric IgG without this activity, and isolated from the same individual, were compared. The IgG complexes had a significantly deviant CD spectrum in the near u.v. region, both before and after dissociation, whereas the monomeric IgG had a normal spectrum.

Immunoglobulins were isolated from the same serum with the use of a specific antiserum against unique determinants in some IgG complexes with RF activity. Both before and after dissociation the CD spectrum in the far u.v. region of these immunoglobulins differed significantly from that of the above-mentioned preparations.

The results confirmed that the structure of IgG in the RF-active complexes differed from that of normal IgG. The immunoglobulins with the unique determinants had, in turn, a structure that was not found in the pool of the RF-active IgG molecules or in normal IgG.

INTRODUCTION

IgG-complexes occur in the sera from individuals with rheumatoid arthritis (RA) (Kunkel *et al.*, 1961; Pope *et al.*, 1975; Roberts-Thomson *et al.*, 1976) and play a major role in the immunopathology of the disease (Ziff, 1973; Zvaifler, 1974). Previously, we isolated IgG complexes with rheumatoid factor (RF) activity from RA sera and found that the IgG in these complexes differed from normal IgG, for example, concerning subclass distribution and content of neuraminic acid (Hansson *et al.*, 1981). Circular dichroism (CD) might be useful for detecting conformational differences between immunoglobulins. Here, the question was whether CD could be used to detect conformational differences between the IgG in the previously mentioned complexes and normal IgG.

The immunogens that elicit the formation of RF *in vivo* are still unknown. Specific rabbit antibodies prepared against unique antigenic determinants in the previously mentioned IgG complexes with RF activity also reacted with some, but not with all, RF-active IgG complexes in other RA sera (Hansson *et al.*, 1981). The question was whether CD could be used to confirm the structural differences between the molecules with the unique determinants and the pool of IgG complexes.

In this investigation, the CD was measured for

IgG complexes with RF activity, for monomeric IgG without RF activity and for immunoglobulins with the unique antigenic determinants, all isolated from one RA serum. The result showed significant differences not only between IgG in the IgG complexes and the monomeric IgG, but also between the immunoglobulins with the unique antigenic determinants and the fraction containing the pool of IgG complexes.

MATERIALS AND METHODS

Serum

The serum (OS) was from a patient with seropositive rheumatoid arthritis (RA) and had been stored at -20°C . It contained 27% IgG complexes ($\geq 9\text{S}$, $< 19\text{S}$).

Isolation of IgG complexes and monomeric IgG

The IgG complexes had RF activity and were isolated by affinity chromatography to immobilized heat-denatured human IgG as described earlier (Hansson *et al.*, 1981). The IgG complexes and the monomeric IgG without affinity to heat-denatured human IgG were further purified by ion-exchange chromatography (DEAE-Sephacel; 0.02 M sodium phosphate, pH 8.0). Monomeric reference IgG was isolated from a commercial IgG preparation for human use (Gamastan, Cutter Laboratories,

Berkeley, California, U.S.A.) by gel filtration (Sephacryl S-300; 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0). Immunoglobulins with unique antigenic determinants (Hansson *et al.*, 1981) were isolated by affinity chromatography to immobilized F(ab')₂γ of specific rabbit antibodies against IgG with RF activity isolated from a homologous serum (BL; Hansson *et al.*, 1981). The *s*-rate distribution of the components in the samples was determined as described before (Hansson *et al.*, 1981).

CD

CD spectra were obtained with a Jasco J-40 A spectropolarimeter at 22°C. The cell length was 1.00 cm between 250 and 320 nm and 0.10 cm below 250 nm. Three solvents were used: 0.012 M sodium phosphate, 0.014 M NaCl, pH 7.3; 0.004 M sodium acetate, pH 5.4; and 0.05 M sodium phosphate, pH 11.0. The samples were filtered before CD analysis through sterile 0.45-μm cellulose acetate membranes. The light absorbancy at 280 nm ($E_{1\%}^{1\text{cm}} 280 \text{ nm}$, = 12.5; Metzger, 1970) was below 1.5 absorbancy units. Furthermore, the protein concentration was determined immediately before CD analysis by the Folin phenol method (Lowry *et al.*, 1951). The mean residue ellipticity (degrees · cm² · dmole⁻¹) was calculated as $[\theta]_i = \frac{\theta \cdot M_0}{c \cdot l}$, where *c* is the protein concentration (g/100 ml); *l* the pathlength in decimeters; *M*₀ the mean residue weight taken as 110, and *θ* the recorded ellipticity in degrees. No Lorentz corrections were made. The errors due to noise were ± 1 degree · cm² · dmole⁻¹ above 250 nm and ± 100 degrees · cm² · dmole⁻¹ at 218 nm. In most cases two separate preparations of each sample were analysed in duplicate. The spectrum that is presented for each sample is the mean value of all the recorded spectra of the sample.

Conclusions about differences between CD spectra were based on either of two calculated degrees of significance:

- (1) The significance was calculated with *t*-statistics (*P* = 0.01) of the majority of the samples.
- (2) The significance was calculated as 2 SD for the CD spectra obtained for IgG complexes, monomeric IgG and reference IgG at pH 11.0, in which cases only one preparation was analysed in duplicate.

The mean residue ellipticities for monomeric (7S) IgM prepared from a macroglobulinaemic IgM (κ) were at pH 7.3, 218 nm: -3165; and at pH 11.0, 218 nm: -3341; 254 nm: +128.7.

The mean residue ellipticities for IgA were at pH 7.3, 218 nm: -3900 (Underdown & Dorrington, 1974) and this value was also used at 218 nm at pH 11.0. At 254 nm at pH 11.0 the value of reference IgG was used.

The mean residue ellipticity of the mixture of IgG (or IgG complexes) and IgM and IgA were calculated from the previously mentioned data, according to Dorrington & Smith (1972). The standard deviations given for the mean residue ellipticity of the mixtures in Table 1 are those obtained experimentally for reference IgG and IgG complexes, respectively.

Protein determinations

Human serum proteins were identified by immunoelectrophoresis (Sheiddegger, 1955) and double immunodiffusion (Ouchterlony, 1962) with monospecific rabbit antisera. IgA was quantified with the use of laser turbidimetry (Blom & Hjörne, 1976) and IgM by single immunodiffusion (Hansson *et al.*, 1981).

RESULTS

The CD (195–320 nm) of IgG with RF activity (90% IgG complexes and 10% monomeric IgG) was compared with the CD of monomeric IgG without RF activity from the same patient and both were compared with a reference IgG isolated from a commercial IgG preparation. The IgG samples used were immunologically

Table 1. Comparison of the mean residue ellipticities ± 1 SD (degrees · cm² · dmole⁻¹) of the immunoglobulins with the unique determinants and two reference mixtures

Sample	218 nm		254 nm
	pH 7.3	pH 11.0	pH 11.0
Preparation with unique determinants	-3882 ± 377	-3869 ± 256	97.2 ± 4.0
Normal IgG reference mixture ^a	-2905 ± 242	-3042 ± 64	59.8 ± 1.6
IgG complex reference mixture ^b	-2829 ± 137	3042 ± 33	54.3 ± 1.6

The reference CD spectra were calculated as follows:

^aNormal IgG reference mixture: $0.6 [\theta]_{\lambda}^{\text{normal IgG}} + 0.3 [\theta]_{\lambda}^{\text{IgM}} + 0.1 [\theta]_{\lambda}^{\text{IgA}}$.

^bIgG complex reference mixture: $0.6 [\theta]_{\lambda}^{\text{IgG complexes}} + 0.3 [\theta]_{\lambda}^{\text{IgM}} + 0.1 [\theta]_{\lambda}^{\text{IgA}}$.

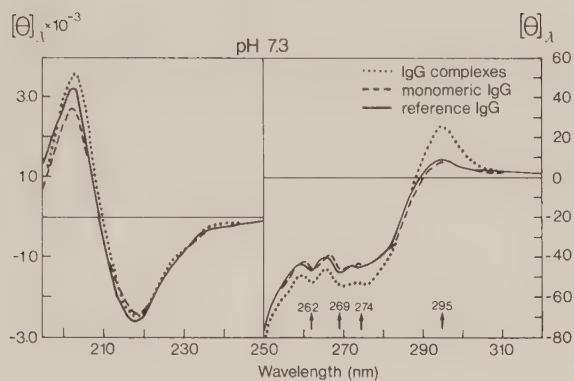


Fig. 1. CD, at pH 7.3, of IgG complexes with RF activity and monomeric IgG without RF activity and isolated from one RA serum compared with a reference IgG. The arrows indicate significant differences between the IgG complexes and the reference IgG. The standard deviations were 137–242 degrees · cm² · dmole⁻¹ at 218 nm and 0.6–3.5 (median value 1.7) degrees · cm² · dmole⁻¹ above 250 nm.

pure and contained polyclonal IgG with kappa and lambda light chains. The CD spectrum of the non-dissociated IgG complexes at pH 7.3 was quantitatively different from the corresponding CD spectra, which were identical, of the other two IgG preparations at all transitions in the near u.v. region (Fig. 1). The CD spectra obtained at pH 5.4 (Fig. 2), when the IgG complexes had dissociated, were similar to those obtained at pH 7.3, but the positive ellipticity at 295 nm was less for the dissociated complexes than for non-dissociated complexes (compare Figs 1 and 2).

Ultracentrifugal analysis showed that the IgG complexes also dissociated at pH 11.0. The CD spectra of the three IgG preparations at this pH (Fig. 3) differed qualitatively and quantitatively from those obtained at pH 7.3. It was of interest

to note that now the reference IgG had mean residue ellipticities between those of IgG from dissociated complexes and native monomeric IgG, which together constituted the IgG pool of the RA serum.

One immunoglobulin preparation containing unique determinants was isolated by affinity chromatography of the RA serum to the immobilized F(ab')₂γ of a specific rabbit antiserum against IgG complexes with RF activity that had been isolated from a homologous serum. This preparation consisted of 40% 7S and 60% complexes (≥ 9S, < 19S) and contained not only IgG, but also 30% IgM (< 19S), 10% IgA and less than 5% Clq. The CD obtained at pH 7.3 and 11.0 was compared with the CD of two reference mixtures at the same pH (Table 1). The reference mixtures were normal

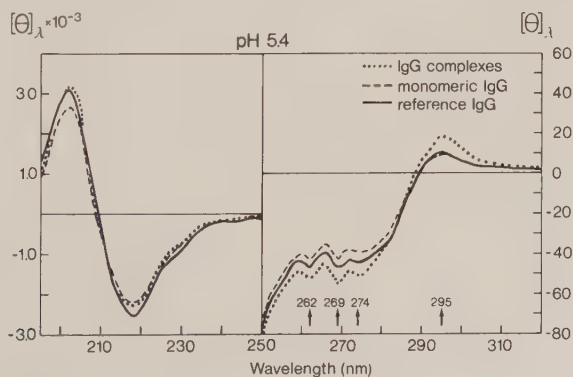


Fig. 2. CD, at pH 5.4, of dissociated IgG complexes with RF activity and monomeric IgG without RF activity and isolated from one RA serum compared with a reference IgG. The arrows indicate significant differences between the dissociated IgG complexes and the reference IgG. The standard deviations were 33–192 degrees · cm² · dmole⁻¹ at 218 nm and 0.4–1.6 (median value 1.0) degrees · cm² · dmole⁻¹ above 250 nm.

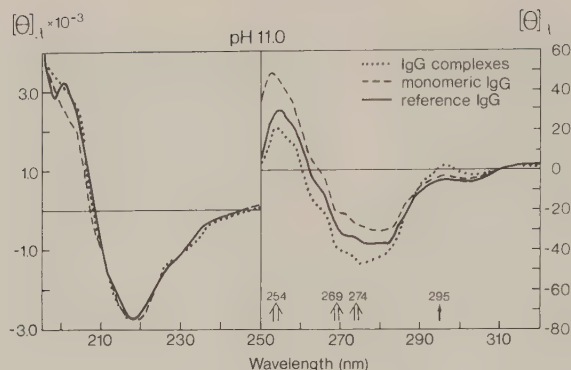


Fig. 3. CD, at pH 11.0, of dissociated IgG complexes with RF activity and monomeric IgG without RF activity and isolated from one RA serum compared with a reference IgG. The solid arrow indicates a significant difference between the dissociated IgG complexes and the reference IgG. The blank arrows indicate significant differences between the dissociated IgG complexes, the monomeric IgG and the reference IgG. The standard deviations were $33\text{--}75 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmole}^{-1}$ at 218 nm and $0.1\text{--}4.5$ (median value 0.8) $\text{degrees} \cdot \text{cm}^2 \cdot \text{dmole}^{-1}$ above 250 nm.

IgG and IgG complexes, respectively, and 7S IgM and IgA, in the same proportions as in the preparation. At pH 7.3 the CD of the preparation differed significantly from that of the two reference mixtures at 218 nm. At pH 11.0, that is, when the complexes had dissociated, the ellipticity of the preparation at 218 nm was still significantly lower than those of the two reference mixtures, which were similar. At pH 11.0, however, differences in ellipticity also occurred at 254 nm. Thus, the structure of the components with unique determinants reflected by the ellipticity at 218 nm at a neutral and an alkaline pH and at 254 nm at an alkaline pH, differed from that of the immunoglobulins in the two reference mixtures.

DISCUSSION

The agreement between our results obtained with the reference IgG and those obtained with IgG by others (Doi & Jirgensons, 1970; Dorrington & Smith, 1972) at different pH levels accentuated the significance of the differences between our reference IgG and the IgG preparations from the RA serum.

Differences between the IgG complexes and the reference IgG were obtained at several transitions in the near u.v. region. The transition at 295 nm has been attributed to tryptophan and the other transitions in the near u.v. region more generally to aromatic amino acids and disulphide bonds in asymmetric environments (Dorrington & Smith, 1972). Thus, tryptophan, other aromatic amino acids and disulphide bonds in asymmetric

environments contributed to the abnormal structure of IgG in the complexes. The absorption band at 295 nm was reduced when the IgG complexes had dissociated and reflected mainly the optical activity of tryptophan.

Small differences have been reported in the CD spectra of IgG isolated from RA serum and normal IgG (Johnson *et al.*, 1974) at 280 nm at neutral pH but the correlation of these differences with RF activity are still obscure. In our study, the IgG complexes with RF activity isolated from RA serum showed quantitative differences at several transitions in the near U.V. region compared to the reference IgG, whereas IgG without RF activity isolated from RA serum had a normal CD spectrum at a neutral pH. Some IgG complexes had not been formed due to self-association of RF (Hansson *et al.*, 1981). It therefore remains to be investigated whether the differences in CD spectra are related to the structure of IgG with or without RF activity.

The ellipticity at 218 nm of the immunoglobulins with common antigenic determinants was 20% more negative than that of the corresponding mixtures of normal IgG (or IgG complexes), IgM and IgA. Negative ellipticity values at 218 nm, deviating as much as 30% from the value of normal rabbit IgG have been reported for individual specifically purified rabbit antibodies (Bear *et al.*, 1974). The components with unique antigenic determinants might also comprise a population with special antibody properties since the specific antiserum reacted with the $F(ab')_2\gamma$ part of the components (Hansson *et al.*, 1981).

The unique antigenic determinants were not related to RF and were only exposed in a minor part of the immunoglobulin complexes with RF activity (Hansson *et al.*, 1981). This was in agreement with the difference between the CD of the components with the unique antigenic determinants and that of the IgG complexes with RF activity from the same patient. It would appear that the unique antigenic determinants were related to immunoglobulins with an abnormal conformation. Whether this conformation was present in unique RF or in immunoglobulins bound to RF remains to be investigated.

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Circular Dichroism of Immune Complexes, IgG and Fab γ with Unique Antigenic Determinants from Rheumatoid Serum

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Uesson, M. & Hansson, U.-B. Circular Dichroism of Immune Complexes, IgG and Fab γ with Unique Antigenic Determinants from Rheumatoid Serum. *Scand. J. Immunol.* **16**, 249–256, 1982.

IgG–IgG and IgG–IgM complexes were isolated from one rheumatoid arthritis (RA) serum by affinity chromatography to immobilized F(ab') $_2\gamma$ of specific antibodies against unique determinants in the complexes. Both IgG and IgM, when isolated from these complexes, contained the unique determinants. The circular dichroism (CD) spectrum of IgG differed from that of normal IgG at neutral pH. At pH 3 both IgG and IgM displayed normal CD spectra, and only one third of the molecules now had affinity for the immobilized ligand. The molecules with affinity at pH 3 exhibited an abnormal CD spectrum at pH 3, and a normal CD spectrum was obtained only of those components that lacked affinity. One-third of the Fab γ isolated from the IgG and IgG complexes with the unique determinants contained the unique determinants that were lacking in the rest of the Fab γ and in all of the Fc γ fragments. The CD of the two Fab γ preparations showed the same principal differences as the CD of the molecules with and without affinity to the ligand at acidic pH.

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Circulating immune complexes occur in serum from individuals with rheumatoid arthritis (RA) [8, 14] and are presumed to play a major role in the immunopathology of the disease [20, 21].

All the immunoglobulins included in the complexes formed *in vivo* must be isolated and characterized before the mechanisms of the complex formation can be clarified. Present concepts are based largely on studies [13] of three RA sera, which contained immune complexes (IC) of intermediate size (>7S; <19S) and were formed by self-association of identical circulating IgG molecules with rheumatoid factor (RF) activity. The self-association implies that these complexes did not contain an antigen or immunogen responsible for the RF production.

Mechanisms other than self-association have also been demonstrated. The IC in the sera of four individuals with RA or hypergamma-

globulinaemia contained different Fab γ fragments, since some Fab γ bound to heat-denatured human IgG and other Fab γ did not [7]. The complexes in one hypergamma-globulinaemic serum were composed of two different subclasses, one of which was RF and bound to the other subclass [6].

We have previously reported the occurrence of common antigenic determinants, not found in normal immunoglobulins, in polyclonal IgG–IgG and IgM–IgG complexes isolated from serum from three individuals with RA or hypergamma-globulinaemia [7]. The unique antigenic determinants were not related to RF activity [7]. Analysis with circular dichroism (CD) showed that the conformation of the immunoglobulins with the unique determinants differed from that of corresponding molecules without these determinants [1]. The question was, however, whether the unique determinants were related to the conformation

of the intact complexes or to the conformation of some or all of the constituent molecules, and, in the latter case, to what protein or part of that protein.

In the present investigation, the constituent molecules of the complexes that contained the unique antigenic determinants were separated from those lacking such determinants, and the CD spectra of the different molecules and of their proteolytic fragments were determined.

MATERIALS AND METHODS

Human serum. Serum from one patient (OS) with RA was used. Sera from a large number of registered blood donors were pooled. The serum OS and the pool were stored at -20°C before use.

Preparation of rabbit antiserum against unique antigenic determinants. Immune complexes, isolated from the RA serum by affinity chromatography to immobilized heat-denatured human IgG as described earlier [7], were used to immunize rabbits bred in our laboratory. They were offspring of rabbits that had successfully produced specific antisera against immune complexes [7]. Immunogen, 1 mg dissolved in 0.5 ml 0.16 M NaCl, 0.1 M glycine, pH 7, was mixed with 0.5 ml complete Freund's adjuvant (Difco, Detroit, Mich., USA) and was injected at several sites behind the scapulae of each rabbit. Booster doses were given in the same manner when required. $\text{F(ab')}_2\gamma$ fragments were isolated from the antiserum pool before absorption. The absence of undigested IgG was checked by analytical ultracentrifugation against a standard human 7S IgG preparation. In this experiment, the protein concentration in the $\text{F(ab')}_2\gamma$ preparation was 10 mg/ml, and control experiments showed that 2% IgG in the sample would be detected. The sedimentation value of the purified $\text{F(ab')}_2\gamma$ fragments was 4.8 S. The $\text{F(ab')}_2\gamma$ fragments were absorbed by repeated affinity chromatography to a pool of normal human serum immobilized [3] to Sepharose CL 4B. The $\text{F(ab')}_2\gamma$ fractions used in this study lacked affinity to this ligand.

Isolation of immune complexes with unique antigenic determinants. The absorbed $\text{F(ab')}_2\gamma$ fragments were immobilized [3] to Sepharose CL 4B using 5.5 mg $\text{F(ab')}_2\gamma$ per 1 ml gel. Two millilitres serum was applied to a column containing 110 ml substituted gel. Sequential elution was performed with 0.075 M Tris, 0.075 M NaCl, 10^{-3} M CaCl_2 , pH 8; 1 M NaCl; and 0.2 M 2-aminobutane, 0.075 M Tris, 10^{-3} M CaCl_2 , pH 11.

In one experiment, the complexes containing the unique determinants were dissolved in 0.1 M glycine-HCl, pH 3, and reappplied to the column equilibrated with the same solvent, followed by sequential elution with 1 M NaCl and 0.2 M 2-aminobutane, pH 11. In another experiment, serum

was applied to the column with Tris, pH 8 followed by sequential elution with glycine-HCl, pH 3; 1 M NaCl; and 0.2 M 2-aminobutane, pH 11. These elution procedures gave equivalent results. The eluates were concentrated in collodium bags by vacuum dialysis against appropriate diluents. The immobilized $\text{F(ab')}_2\gamma$ specific against unique determinants also contained $\text{F(ab')}_2\gamma$ without known antibody activity. The non-specific binding of normal human serum proteins to the column was less than 2%. This was equivalent to the non-specific binding of OS serum proteins to a corresponding column containing an equivalent amount of exclusively normal rabbit $\text{F(ab')}_2\gamma$ fragments without known antibody activity. The specific binding of the components in serum OS to the immobilized $\text{F(ab')}_2\gamma$ of the specific antibodies was fivefold that of the non-specific binding. Similar amounts of specific binding were obtained for the serum proteins in three homologous sera, which indicated that the $\text{F(ab')}_2\gamma$ were not directed against individually specific determinants in the RA serum.

Isolation of IgG and IgM from immune complexes. Isolated immune complexes containing the unique determinants were dissociated by dialysis against 0.2 M 2-aminobutane, 0.075 M Tris, 10^{-3} M CaCl_2 , pH 11, followed by separation of IgG and IgM on a column of Sephacryl S-300 equilibrated in the same buffer.

Isolation of IgG fragments. $\text{F(ab')}_2\gamma$ fragments obtained by pepsin digestion [18] of rabbit IgG were isolated by repeated gel filtration (Sephacryl S-200; 0.05 M sodium phosphate, 0.5 M NaCl, pH 7). Fab_γ and Fc_γ fragments obtained by papain digestion [15] of human IgG were separated by ion-exchange chromatography (DEAE-Sephacel). Fab_γ were eluted with 0.02 M sodium phosphate, pH 8, and Fc_γ with 0.2 M sodium phosphate, pH 6. The digestions and the purity of the isolated fragments were checked by ultracentrifugal analysis, agarose gel electrophoresis, and immunoelectrophoresis or double immunodiffusion against appropriate monospecific antisera.

Isolation of IgG. Monomeric normal human IgG was isolated from a commercial IgG preparation (Gamastan, Cutter Laboratories, Calif., USA) by ion-exchange chromatography (DEAE-Sephacel; 0.02 M sodium phosphate, pH 8) followed by gel filtration (Sephacryl S-300; 0.05 M sodium phosphate, 0.5 M NaCl, pH 7). Rabbit IgG was prepared from serum by salting out in 1.84 M $(\text{NH}_4)_2\text{SO}_4$ overnight at 4°C . The washed precipitates were dissolved in, and dialysed against, 0.16 M NaCl. The preparation was then applied to a column containing DEAE-Sephacel, from which the IgG was eluted with 0.02 M sodium phosphate, pH 8.

Isolation of normal human IgM. Normal human IgM was isolated from a normal serum pool by affinity chromatography on a column containing rabbit $\text{F(ab')}_2\gamma$ anti-human IgM Fc_μ immobilized [3] to Sepharose CL 4B. IgM was eluted with 0.2 M 2-aminobutane, 0.075 M Tris, 10^{-3} M CaCl_2 , pH 11.

Circular dichroism. CD spectra were obtained

with a Jasco J-40 A spectropolarimeter at 22°C. The cell length was 1.00 cm between 250 and 320 nm and 0.10 cm below 250 nm. The IgG preparations were analysed in a neutral buffer with a low ionic strength (0.012 M sodium phosphate, 0.014 M NaCl, pH 7) to compare the results obtained in this study with those obtained earlier [1]. All other immunoglobulin preparations were analysed in solvents with a higher ionic strength: 0.05 M sodium phosphate, 0.5 M NaCl, 0.1 M glycine, pH 7, or 0.1 M glycine-HCl, pH 3.

The samples were filtered before CD analysis through sterile 0.45- μ m cellulose acetate membranes, and the protein concentration was determined immediately before CD analysis by the Folin phenol method [9]. All samples were stable during CD analysis in the solvents used, and no precipitation occurred. The light absorbancy at 280 nm was below 1.5 absorbancy units. The mean residue ellipticity (degrees $\cdot$ cm² $\cdot$ dmol⁻¹) was calculated as $[\theta]_{\lambda} = (\theta \cdot M_0) / (c \cdot l)$, where c is the protein concentration (g/100 ml); l the pathlength in decimeters; M_0 the mean residue weight taken as 110, and θ the recorded ellipticity in degrees. No Lorenz corrections were made. The errors due to noise were ± 1 degree $\cdot$ cm² $\cdot$ dmol⁻¹ above 250 nm and ± 100 degrees $\cdot$ cm² $\cdot$ dmol⁻¹ at 218 nm. All samples were analysed in duplicate. Conclusions about differences between CD spectra were based on the significance calculated as 2 SD for each CD spectrum. The mean residue ellipticities for mixtures of normal IgG and IgM were calculated [4] from the CD spectra obtained in this study. The standard deviations for the mean residue ellipticity of the mixtures in Table I were those experimentally obtained for normal IgG and IgM, respectively.

Electrophoretic and immunodiffusion methods. Agarose (1%) gel electrophoresis was run in 0.075 M barbital buffer made 2 mM with regard to calcium lactate at pH 8.6. Human serum proteins were identified by immunoelectrophoresis [16] and double immunodiffusion [11] with monospecific antisera.

Ultracentrifugal analysis. Ultracentrifugal analysis was done with an An-D rotor at 59,780 rpm (259,700

g) in a Beckman Analytical Ultracentrifuge Model E with Schlieren optics at 20°C as described previously [7]. The molecules that had affinity and those that lacked affinity to the immobilized ligand in 0.1 M glycine-HCl at pH 3 were analysed in 0.1 M glycine-HCl, pH 3. All other samples were analysed in 0.2 M 2-aminobutane, 0.075 M Tris, 10⁻³ M CaCl₂, pH 11, and/or in 0.05 M sodium phosphate, 0.5 M NaCl, 0.1 M glycine, pH 7.

RESULTS

The rheumatoid serum contained 4% rapidly sedimenting complexes (RC; >19S) and 22% IC ($\geq 9S$; <19S). All of the RC and about 20% of the IC contained the unique determinants and bound to the immobilized F(ab')₂ γ of the specific rabbit antibodies. This preparation (at a concentration of 10 mg/ml) contained 60% RC and 40% IC at pH 7 and 85% 7S and 15% 19S at pH 11. The preparation was gel filtered at pH 11, and the 7S and 19S components were separated. The 7S component contained mainly IgG and the 19S component mainly IgM, and thus the rapidly sedimenting complexes were composed of IgG and 19S IgM and the intermediate complexes of IgG. At pH 7, 80–90% of the IgG formed IC, whereas the IgM remained as a 19S component. When reappplied to the immobilized ligand at neutral pH, the IgG bound quantitatively, but because of the content of IgG complexes the possibility could not be excluded that part of the IgG within the complexes lacked affinity for the ligand. Under the same conditions, the isolated 19S IgM bound quantitatively to the ligand.

The CD (210–320 nm) of the isolated IgG

TABLE I. Comparison of the mean residue ellipticities ± 2 SD (degrees $\cdot$ cm² $\cdot$ dmol⁻¹) of molecules with unique determinants at pH 3, of molecules without unique determinants at pH 3, and of two reference mixtures. The CD was recorded in 0.1 M glycine-HCl, pH 3

Sample	218 nm	262 nm	269 nm	275 nm	295 nm
Molecules without unique determinants at pH 3					
Experimental value	-3235 $\pm$ 310	-37.7 $\pm$ 2.9	-41.4 $\pm$ 0.3	-39.9 $\pm$ 0.2	11.4 $\pm$ 0.4
Reference mixture*	-2966 $\pm$ 97	-43.0 $\pm$ 7.7	-42.8 $\pm$ 3.7	-36.5 $\pm$ 4.1	15.0 $\pm$ 2.0
Molecules with unique determinants at pH 3					
Experimental value	-3936 $\pm$ 1221	-18.7 $\pm$ 2.2	-21.3 $\pm$ 1.0	-23.8 $\pm$ 5.0	6.4 $\pm$ 0.8
Reference mixture†	-3130 $\pm$ 97	-33.4 $\pm$ 7.7	-32.8 $\pm$ 3.7	-28.9 $\pm$ 5.9	9.4 $\pm$ 2.0

The reference CD spectra were calculated as follows: * 0.9 $[\theta]_{\lambda}$ normal IgG + 0.1 $[\theta]_{\lambda}$ normal IgM. † 0.5 $[\theta]_{\lambda}$ normal IgG + 0.5 $[\theta]_{\lambda}$ normal IgM.

and of normal IgG were compared. At pH 7 the two IgG gave different CD at all transitions in the near ultraviolet region (Fig. 1). However, the spectra were similar in the same region at pH 3 (Fig. 2); that is, the conformation of the IgG with the unique determinants was 'normalized' when it was dissolved in glycine-HCl, pH 3.

The CD (210–320 nm) of IgM containing the unique determinants and normal IgM were also similar at all transitions when both were dissolved in 0.1 M glycine-HCl at pH 3 (Fig. 3). The unique IgM precipitated under the conditions used for CD analysis at neutral pH, and no CD spectrum was recorded at this pH.

The complexes with affinity for the immobilized ligand at neutral pH were dissolved in 0.1 M glycine-HCl, pH 3, and reapplied to the column equilibrated with the same solvent. Two-thirds of the components (90% 7S and 10% 19S) were eluted with glycine-HCl at pH 3, whereas one-third of the components (50% 7S and 50% 19S) bound to the column at this pH and thus contained the unique determinants at pH 3. The bound components

were eluted with 2-aminobutane at pH 11. Both fractions contained IgG and IgM. The CD spectra (210–320 nm) of the two fractions, determined at pH 3, are given in Fig. 4. Differences were found at all transitions in the near ultraviolet region. The CD was also compared with the calculated CD for the corresponding mixtures of normal IgG and IgM in 0.1 M glycine-HCl, pH 3. The population without unique determinants at pH 3 had CD spectra similar to those of the normal proteins except at 295 nm, whereas the population with unique determinants at pH 3 showed marked deviations from the normal proteins at 262, 269 and 295 nm (Table I).

Fab γ and Fc γ fragments were isolated from the IgG and IgG complexes that contained the unique antigenic determinants. One-third of the Fab γ had affinity for the immobilized ligand at neutral pH and thus contained the unique determinants. All the Fc γ lacked affinity for the ligand. The CD spectra of the two Fab γ populations were quantitatively different at all transitions in the near ultraviolet region (Fig. 5) and exhibited the same principal differences as observed for molecules

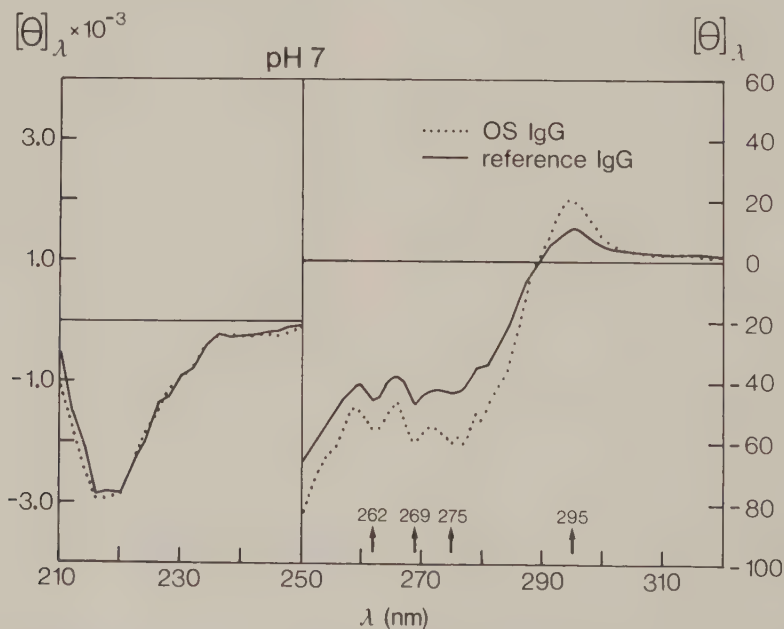


FIG. 1. CD at pH 7 of OS IgG with unique determinants, compared with a reference IgG. The arrows indicate significant differences between the unique IgG and the reference IgG. The standard deviations (2 SD) were 5–63 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ at 218 nm and 0.9–4.4 (median value, 2.5) degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ above 250 nm.

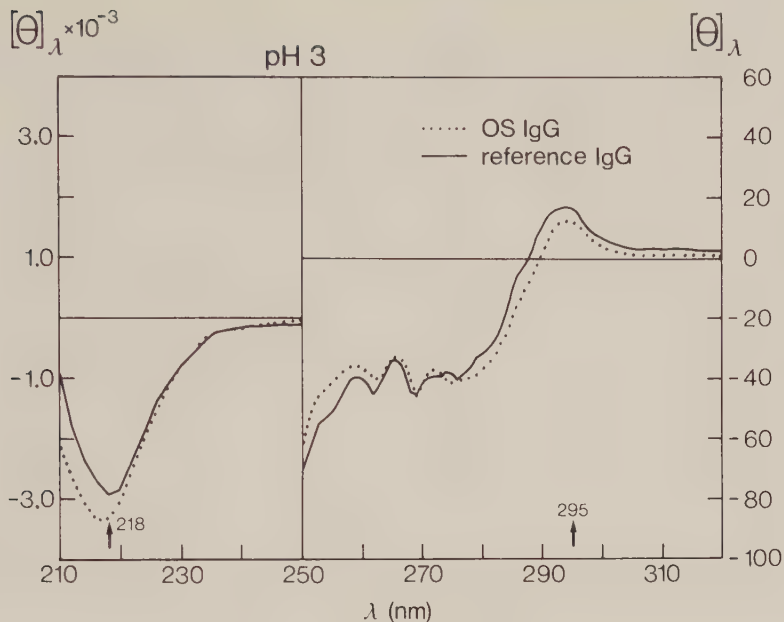


FIG. 2. CD at pH 3 of OS IgG with unique determinants, compared with a reference IgG. The arrows indicate significant differences (2 SD) were 70 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ at 218 nm and 0.7–7.7 (median value, 2.1) degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ above 250 nm.

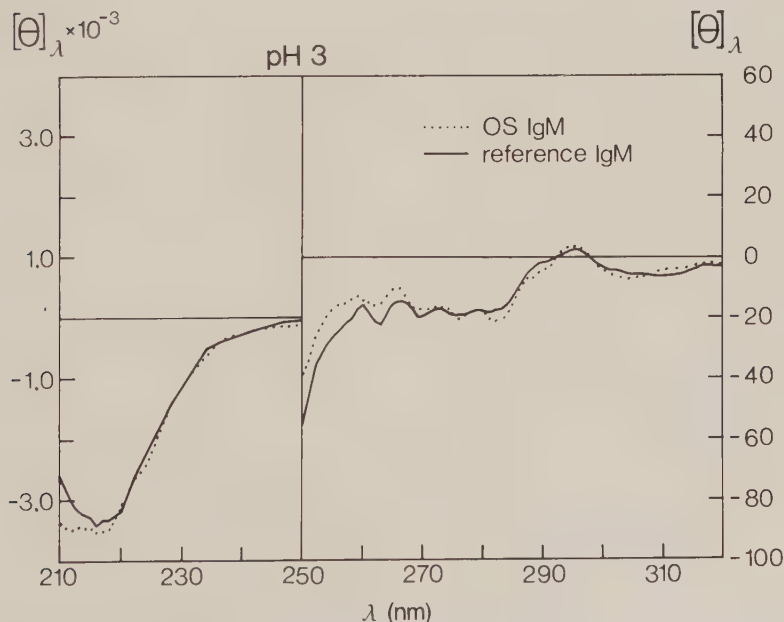


FIG. 3. CD at pH 3 of OS IgM with unique determinants, compared with a reference IgM. The standard deviations (2 SD) were 97–117 degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ at 218 nm and 1.3–10.6 (median value, 4.1) degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ above 250 nm.

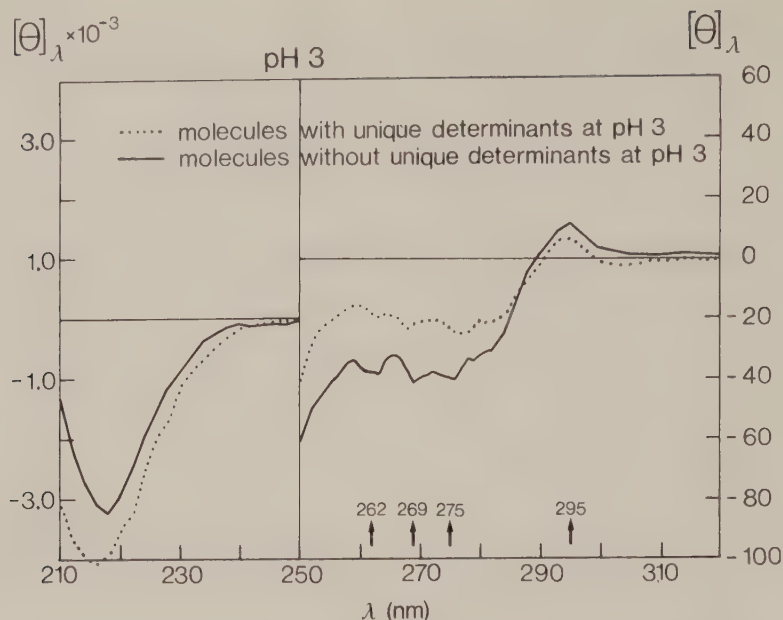


FIG. 4. CD at pH 3 of molecules with unique determinants at pH 3, compared with molecules without unique determinants at the same pH. The arrows indicate significant differences between the CD spectra. The standard deviations (2 SD) were 310–1221 degrees·cm²·dmol⁻¹ at 218 nm and 0.2–5.0 (median value, 1.0) degrees·cm²·dmol⁻¹ above 250 nm.

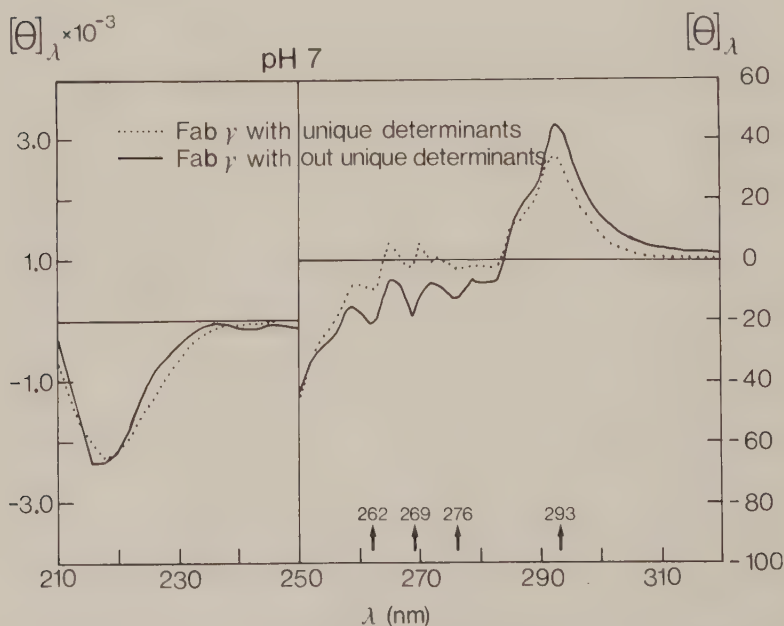


FIG. 5. CD at pH 7 of Fab γ with unique determinants, compared with Fab γ without unique determinants. The arrows indicate significant differences between the CD spectra. The standard deviations (2 SD) were 0–380 degrees·cm²·dmol⁻¹ at 218 nm and 0.6–4.0 (median value, 2.4) degrees·cm²·dmol⁻¹ above 250 nm.

with and without unique determinants at acidic pH (compare Figs. 4 and 5).

DISCUSSION

The CD spectrum of the unique IgG complexes was different from that of a reference IgG at all transitions in the near ultraviolet region at neutral pH but similar to the one obtained earlier [1] for all IgG complexes in the same RA serum. The abnormal CD spectrum obtained for the latter preparation was thus related to the presence of IgG with unique determinants within the pool.

When dissolved in glycine-HCl at pH 3, contrary to when dissolved in phosphate buffer at pH 7, the IgG with the unique determinants showed a normal CD spectrum. The IgG complexes dissociated in the acidic medium. The ellipticity at 295 nm has been shown to be reduced when IgG complexes are dissociated, whereas the ellipticity at other wavelengths remains unchanged [1]. Thus, the 'normalization' of the CD spectrum observed in glycine-HCl, pH 3, could not only be explained by a dissociation of IgG molecules. Transitions at 262, 269 and 275 nm have generally been attributed to aromatic amino acids and disulphide bonds in asymmetric environments [4]. The results therefore indicated that some conformation related to non-polar residues was 'normalized' in the acidic and polar glycine solution. The question was whether this conformation was related to that of the unique determinants.

As had been the case for IgG with unique determinants, a normal CD spectrum was also obtained for the corresponding IgM at pH 3. It was therefore of interest that, when dissolved in glycine-HCl, pH 3, one-third of the components still had affinity to the immobilized F(ab')₂ γ of the specific antibodies. This showed that only a portion of IgG and IgM contained the unique determinants and that these determinants were also exposed in the glycine pH 3 solvent. CD analysis at pH 3 showed that only the small portion of IgG and IgM with unique determinants had an abnormal CD spectrum at pH 3. Conformational differences between molecules with and without the unique determinants could thus also be observed at pH 3. This meant that the 'normal-

ization' of the CD spectra observed earlier at pH 3 was not related to the conformation of the unique determinants. That aromatic or other non-polar groups were of importance for the interactions between the unique molecules and the F(ab')₂ γ of the specific rabbit antibodies was demonstrated by the fact that elution from the ligand could be performed with the partly non-polar 2-aminobutane solvent but not with the more polar glycine solution.

One-third of the Fab γ fragments, isolated from the purified IgG fraction, had affinity to the immobilized ligand at neutral pH. IgG has two identical Fab γ parts [19]. The IgG preparation thus contained two different populations of IgG molecules, one of which had unique determinants. CD analysis at neutral pH of the Fab γ with and without unique determinants demonstrated the same principal deviations as found for IgG and IgM with and without unique determinants at pH 3. The ratio of molecules with unique determinants was the same for Fab γ at pH 7 and the intact molecules at pH 3. Our conclusion was that the molecules with unique Fab γ were those that had affinity at pH 3. The presence of the unique determinants was correlated to the ellipticity at the transitions in the near ultraviolet region that reflect mainly the optical activity of tryptophan, other aromatic amino acids, and disulphide bonds.

The occurrence of similar idiotypes in various polyclonal and monoclonal RF [2, 5, 12] indicates a restriction in the conformation of the RF binding site of the immunogenic/antigenic IgG. It has recently been shown [10] that anti-idiotypic antibodies can possess antigenic determinants that are related to determinants on the original antigen. This may be related to autoimmune disease and, for example, to the assumption that antibodies to insulin receptors in diabetic patients may be anti-idiotypic antibodies, directed against anti-insulin antibodies [17]. On the basis of these findings the following model might offer one explanation for the results obtained in the present investigation: RF may contain idiotypic determinants in the antigen-binding site complementary to certain determinants on an IgG antigen occurring in serum. At the same time autologous anti-idiotypic antibodies against the idiotypic determinants in RF are present. The deter-

minants present on the IgG antigen might be related to those of the anti-idiotypic antibodies. There are indications that the unique antigenic determinants studied here were not related to RF activity (M. Uesson, unpublished observations, 1982). This model then suggests that the Fab γ with unique determinants found in this investigation might originate from an autologous IgG antigen or from an autologous anti-idiotypic IgG.

The location of the unique determinants in the Fab γ fragments and the biological activity of these Fab γ will be investigated. From the results described here, it would appear that the unique Fab γ fragments originated from molecules bound to RF, rather than from RF itself.

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